Identification of candidate tumor suppressor genes' loci on chromosome 9 associated with the development of arsenic induced urinary bladder cancer in West Bengal, India.

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Abstract

This study aimed to analyse the impact of arsenic as key etiological factors in the development of bladder cancer in a population of West Bengal inhabiting mainly along arsenic affected regions of the Gangetic belts. Based on previous reports on chromosome 9 aberration in bladder tumorigenesis, alterations of selected genes residing on the chromosome and related molecular pathways has also been aimed to analyse. To fulfil the objectives, arsenic content in tumors were detected and associated with proliferation potential (PF), clinicopathological parameters and patient outcomes. Selection of genes from chromosome 9 were done based on their high deletion frequencies exclusively in arsenic high tumors evident from our array data. Detailed molecular alteration (deletion, promoter methylation, mRNA/protein level expression) of selected genes were performed following standard methods and, the data were associated with tumor arsenic and different disease related parameters. From our data, accumulation of arsenic in tumor tissue and its adverse impact on aggressive tumor phenotypes (high PF), advanced clinicopathological parameters and adverse patient outcomes was evident, suggesting arsenic as a major risk factor in bladder carcinogenesis of the studied population. Among studied genes and related molecular pathways, significant association of *SH3GL2* (chr.9p22) mediated EGFR homeostasis and *PHF2* (chr.9q22.3) mediated histone demethylase pathways was found with arsenic induced bladder carcinogenesis. Briefly, EGFR homeostasis and p53 mediated transcriptional activity were found to be impaired and arsenic induced inactivation of *SH3GL2* (involved in EGFR internalization) and *PHF2* (removes inhibitory methylation mark on p53 target promoter) were put forward as the underlying mechanisms. In our study including both non-muscle and muscle invasive bladder cancer, involvement of the said dysregulation in the early stage of bladder carcinogenesis was evident. In other studied pathways like G1/S cell cycle transition, intra-strand crosslink repair, and negative regulation of smoothened signalling, no significant association was seen with arsenic induced bladder carcinogenesis as evident from comparable alteration frequencies of the constituent genes like *CDKN2A* and *CDKN2B* (9p21, CDK inhibitors), *FANCC* (9q22.3, component of FA-BRCA complex) and *PTCH1* (9q22.3, negative regulator of smoothened signalling) in both arsenic high and low tumors. In conclusion, chromosome 9 harbours important tumor suppressor genes (TSGs) some of which were inactivated preferentially under influence of arsenic leading to dysregulation of associated molecular pathways playing vital role in early stage of bladder carcinogenesis. Of the TSGs, the diagnostic and prognostic importance of potential candidates should be explored further to develop clinically relevant biomarker for this disease subset.

Introduction

Histological origin –

The Urinary bladder cancer (BLCA) (middle to older-aged disease) develops as a change and uncontrolled growth in the normal urothelial lining forming solid tumor. BLCA are mainly of three subtypes: urothelial (transitional cell) carcinoma, squamous cell carcinoma, and adenocarcinoma; and two rarer types namely sarcoma and small cell carcinoma. Out of various subtypes, 90% of the BLCA are accounted as urothelial carcinoma or UCC. On the basis of its invasive nature, BLCA can be subdivided into three categories: non-invasive (papillary carcinoma and carcinoma in-situ), non-muscle invasive (grown into lamina propria), and muscle-invasive (spread to bladder wall muscle, fatty layer, surrounding tissues or organs).[1]

Epidemiology –

BLCA is the 10th most common cancer worldwide, with 5,73,278 new cases and 2,12,536 deaths have been recorded in 2020. The disease ranks 6th in occurrence in men and 9th leading cause of death due to cancer.[2] Overall survival rate of the disease is 77% (69%, if tumor has not spread outside bladder; 37%, when tumor has spread to lymph nodes and other organs).[3] Urothelium becomes susceptible to malignant transformation after getting uninterrupted exposure to hazardous chemicals including carcinogens through its contact with urine. BLCA has a strong aetiology of tobacco smoking, workplace exposure to aromatic amines, intake of certain medicines and herbal supplements, arsenic through drinking water etc.Type-2 diabetes mellitus and schistosomiasis are also considered as risk factors.[4] Arsenic exposure through drinking water is a well-known cause of BLCA.[5,6,7] Higher incidence rate of BLCA from areas with arsenic-induced black-footed disease[8] and malignant transformation of urothelial tissue upon arsenic exposure has made this association more evident.[9] Epidemiological studies reported link between drinking water arsenic and occurrence of BLCA from different parts of the world like Canada[10], Taiwan[11], Chile[12] etc. Majority of them measured arsenic in drinking water but quantification of the same in bladder tissue is lacking.

Histology–

BLCA can be categorized in different ways. According to World Health Organization, **low-grade** tumors are more similar to normal tissue whereas poorly differentiated tumors are called as **high-grade**. Tumor staging can be clinical (based on physical examination, imaging scans) or pathological (biopsies of tumor tissues after surgery for detailed prognosis). TNM classification is followed for tumor staging. (a) **Stage 0a**: non-invasive papillary urothelial

carcinoma on the inner lining of the organ. (b) **Stage 0is**: found only on the inner lining and not grown either toward the hollow part of bladder or to the muscle layer underneath. (c) **Stage I**: grow through inner lining and into lamina propria but never reached to muscular layer. (d) **Stage II**: tumor in thick muscle layer of bladder but not spread upto surrounding fatty layer. (e) **Stage III**: tumor at perivesical tissue or nearby organ and lymph nodes. (f) **Stage IV**: tumor spread to pelvic or abdominal wall or distal lymph nodes (According to American cancer society medical review, 2019).

Molecular pathogenesis –

In spite of its detection at early stage, high recurrence rate (70%) of BLCA with progression to higher grade and/or stage of the disease signifies the heterogeneous nature and malignant potential of this carcinoma. Appropriate management of invasiveness requires accurate detection and assessment of biological potential of tumor. Although, conventional histopathologic diagnosis provide certain degree of biological characteristics, a detailed profiling of individual tumor to identify accurate molecular markers may guide to understand tumor aggressiveness, disease progression, recurrence and in selection of advanced treatment procedure.

A lot of studies have shown genomic instability in BLCA due to loss of heterozygosity (LOH) of several chromosomes like chromosome 9 at early stages and chromosomes 11, 13, 3, 4, 8, 17 and 18 at later stages of tumor development.[13] Cell cycle regulation gene *TP53* that inhibits G1 to S transition by transcriptionally activating *P21* has been mutated and accumulated in nucleus in bladder carcinoma.[14] Loss of p21 expression has also been reported as an independent predictor of bladder cancer progression.[15] *MDM2* amplification has been reported with an increasing frequency with higher stage and grade of tumor.[16] *CDKN2A* locus has been found to be deleted as hemizygous and homozygous condition.[17] Deregulation of the same locus by deletion, methylation had been associated with worst prognosis.[18] Tumor suppressors like *P53, P21, RB, cyclin E1* and *P27*, in combination, has been shown to predict bladder cancer more accurately.[19] Overexpression of Bcl-2 family protein has been associated with poor prognosis.[20] Bax expression is an independent predictor of a more favourable prognosis in invasive tumor.[21]

Both, microsatellite marker based deletion mapping and array-CGH data has suggested loss of several chromosomal locus like 9p22-23, 9p21-22, 9p11-13, 9q12-13, 9q21-22, 9q31 and 9q33-34 with putative tumor suppressors.[22] LOH at 9p21-22.3 and 9q22-23 are frequent in

tumors of lower grade and stage. Deletion on the long arm of chromosome 9 (9q) is found to be twice as frequent as 9p deletion and termed as early event tumorigenic process.[23] Genetic mapping of cystectomy samples has reported chromosome 9 deletion in normal urothelium adjacent to bladder mucosa with early precursor changes.[24] Loss of heterozygosity (LOH) of markers at 9ptr-p22, 9p21, 9q22.3, 9q33, 9q34 were associated with a significantly shorter recurrence-free survival.[25]

Bladder cancer: an Indian scenario

BLCA ranks 8th among all cancers with 1.9% overall incidence rate (1.5% males and 0.4% in females) according to Indian cancer registry.[26] In India, predominantly two types of cancer registries are followed: population-based cancer registry (PBCR) and hospital-based cancer registry (HBCR). Increasing trends of bladder cancer incidence was evident in major cities in India from 1989 to 2014 with Delhi acquiring the highest position and Kolkata in the third.[27]

Diagnosis, Treatment modalities and patient outcome–

Most common symptoms of bladder cancer is haematuria, irritative voiding symptoms, tumor discoverable by imaging etc. Evaluation of these symptoms involves a number of diagnostic approach.

Cystoscopy and Endoscopic resection: Cystoscopy is a procedure to evaluate the lower urinary tract.[28] If anomalies are found during cystoscopy, endoscopic resection or transurethral resection of bladder tumor (TURBT) is recommended. TURBT is a diagnostic as well as potential therapeutic approach, basically resects visible tumors and performs tumor staging. Sometimes, repeat TURBT is recommended in case of incomplete resection, or pathologic high grade Ta/T1 tumors[29] and it may also lead to poor cancer-specific survival.[30]

Treatment of bladder cancer is very much based on the tumor's clinical stage after first diagnosis. (i) Bacille Calmette-Guerin (BCG) therapy: Patients are treated with a live, attenuated form of *Mycobacterium bovis* that are internalized, presented, induced cell-mediated innate and adaptive immune response followed by killing of cancerous cells.[31] Although BCG improves progression-free and recurrence-free survival in patients compared to chemotherapy, it poses some adverse physiological condition such as cystitis, malaise, severe sepsis after systemic absorption, even BCG-unresponsive disease.[32]

Chemotherapy – A single dose of chemotherapeutic drugs has been administered intravesically after TURBT. However, technique is expensive and efficacy depends on drugs used in treatment.[32]

Radiation therapy –High-energy x-rays is used to stop and destroy cancer cells from developing alone or in conjunction with chemotherapy following TURBT.

But each of the diagnostic and therapeutic approaches has their own drawbacks. Detection of bladder cancer is mainly dependent on removal of tumor via TURBT (an invasive method) and evaluation of histopathological staging needs well established hospital set-up and expertise. BCG therapy has complete dependencies on individuals' immune response while chemotherapy and radiotherapy has a huge fatal side effects to the patients. A non-invasive, population-based screening strategy is of urgent need for at least the detection of the disease by general health workers in rural areas which requires identification of potent molecular markers.

Bladder cancer: a rising health problem in West Bengal -

West Bengal comes under the high risk zones of BLCA incidence in respect to lifestyle and environmental factors. According to a report on arsenic contamination of underground water by School of Environmental Studies[33], districts of West Bengal can be categorized into three discrete areas; severely affected (arsenic concentrations >50 μg/L), mildly affected (11-50 μg/L) and arsenic safe (<10 μg/L). Apart from arsenic exposure, tobacco smoking habit is also very common among Eastern Indian population.[34] Although, tobacco smoking is considered as the most common risk factors, arsenic exposure seems to be equally important from the existing evidence.[35]

Gross alteration of regions on chromosome 9p&q that harbours a number of potential tumor suppressor genes (TSGs) were reported from previous investigations from different countries. Moreover, high frequency of copy number variation of both 9p&q in BLCA were found in association with arsenic. Till date no detailed study of genes presenting deleted/amplified regions of chromosome 9p&q has been done in association with BLCA. In Indian perspective especially in West Bengal, background data in this respect is totally missing up to the present state of knowledge.

On the basis of aforesaid backdrop present hospital based study aimed to focus on following objectives-

Objective:

1. Analysis of key etiological factors with special reference to arsenic associated with development of bladder cancer in West Bengal.

2. High resolution copy number variation analysis (CNV) for identification of frequently altered (deletion/amplification) regions of chromosome 9p and 9q associated with development of urinary bladder cancer.

3. Promoter methylation followed by expression analysis of candidate genes located in the frequently altered chromosomal regions.

Methodology:

Study design and recruitment of subjects:

This hospital based study was conducted in Department of Urology of Institute of Postgraduate Medical Education and Research (IPGME&R) after taking approval from the institutional ethical committee of respective hospital and written consent from the patients willing to take part in this study. Patients aged between 18-70 years with primary bladder tumor and not receiving any treatment before operation were included in the study; patients with metastatic bladder tumor and/or receiving radio/chemotherapy before operation were excluded.

Sample and setting:

A total of 214 participants fulfilling the criteria were included for sample collection during the study period [November 2016- March 2020]. In general, a part of freshly operated tumor tissues obtained from TURBT were collected in 10% formalin and the other were frozen immediately at -80^0C. Blood samples were stored at -80^0C. Normal urothelium adjacent to the tumor were available from 12 patients. Tumor stage and grade were detected according to TNM classification under the guidance of respective expert; clinicopathological and etiological details of patients were provided by the hospital authority. Details of samples used in different experiments were tabulated in Figure 1.

Detection of total arsenic by Atomic Absorption Spectrometry:

Total arsenic was measured in bladder tumors and drinking water of patients with the Atomic Absorption Spectrophotometer Duo (Agilent Technologies, CA, USA) equipped with vapour

generation assembly (Agilent VGA77) following the standard protocol.[36] For tumor, briefly 0.1-0.5g of bladder tissue was acid digested (2 HNO_3: 1 $HCLO_4$ mixture) and diluted with double-distilled water up to final volume of 50ml, stored at 4^0C. For instrument calibration, arsenic standards of 5, 10, 20, 40 ppb (parts per billion) were used. Arsenic detection was done using 189-nm wavelengths.

Microdisscetion, Tumor cell enrichment and Isolation of genomic DNA:

Tumor samples were first cryo-sectioned to micro-dissect normal contaminant cells. Tumor tissues containing >70% of tumor cells were selected for DNA isolation along with peripheral blood lymphocyte (PBL) by phenol- chloroform method.[37] Concentration and purity of DNA were checked by measuring absorbance at 260 and 280nm in nanodrop.[38]

Bioinformatical analysis:

Copy Number Variation (CNV) analysis data of the genes were obtained from The Cancer Genome Atlas (TCGA) database using TCGA tools, namely UCSC Xena (www.xena.ucsc.edu). The average normalized log ratio cut-off was fixed at 0.0- 0.25, values greater and lower than cut-off were considered as amplification and deletion, respectively. To examine the expression of selected genes in primary bladder tumor and adjacent normal tissue and among different tumor stages of bladder tumor samples, GEO datasets [GSE13507 (n=174), GSE128959 (n= 198)] were selected from GEO NCBI database. The samples were grouped according to muscle-invasive properties of tumor stages, NMIBC (Ta- T1) and MIBC (T2- T4) and analysed with GEO2R tool to find out differentially expressed gene that are represented in volcano plot. Red and blue dots in the volcano plot represent significant differentially overexpressed and under-expressed genes (adj. $p< 0.05$), respectively.

Copy Number Variation (CNV) analysis:

Deletion analysis by microsatellite/ exonic markers:

This was done by two ways (i) <u>microsatellite based deletion mapping</u>: Briefly, standard polymerase chain reaction (PCR) was performed using [γ-p32] ATP-(specific gravity 3000ci/mmol) labelled forward primer using T4-polynucleotide kinase. Amplified PCR products were electrophoresed on 7% polyacrylamide urea gel containing 8M urea at 35W and auto radiographed at -80^0C. Scoring of loss of heterozygosity (LOH) and molecular size alterations (MA) was done according to Dasgupta et al, 2019.[39] (ii) <u>Analysis by differential PCR (dPCR) method using Exonic marker</u>: PCR was performed using D9S104 as a control

marker. Deletion and retention of alleles were calculated as described by Ichimura et al.[40] Primers used in deletion analysis were tabulated below (table 1):

Table 1: Details of primers used in deletion analysis.

Gene	Marker	Primer	Amplicon size (bp)	Tm (^{0}C)
SH3GL2	SH3GL2 Ex9	F 5'CCGAGCTCTGTACGACTTTG 3' R 5' GCCAGCCAGCATAACATC 3'	193bp	56^{0}C
p14/p16	CDKN2A Ex2	F 5'CTTCCTTTCCGTCATGCC 3' R 5'CTTTGGAAGCTCTCAGGG 3'	412bp	56^{0}C
p15	CDKN2B Ex1	F 5' AAGAGTGTCGTTAAGTTTACG 3' R 5' ACATCGGCGATCTAGGTTCCA 3'	315bp	60^{0}C
FANCC	D9S1958	F 5' GGAGTAATTAGAAGGAGGGAGAGG 3' R 5' CCACTCCTACCTCGCCAG 3'	149bp	60^{0}C
PTCH1	PTCH-CGG	F 5' AGCGGCTGGTCTGTCAAC 3' R 5' TACCAGCCGAGGCCATGT 3'	181bp	62^{0}C
	PTCH Ex22	F 5' CAGGTAAATGGACAAGAAC 3' R 5' CTGTGTGATGTGCTGCTC 3'	403bp	55^{0}C
PHF2	D9S196	F 5' GGGATTACACCTCAAAACCA 3' R 5' ACCACACTGCGGGACTT 3'	258bp	58^{0}C
	PHF2 Ex18	F 5' ACTCCTGCCTGCAGACCAC 3' R 5' CCTGCTCTTCCTCGTAGTCG 3'	168bp	58^{0}C
EGFR	EGFR Ex20	F 5' TCTGGCCACCATGCGAAGC 3' R 5' GGATCCTGGCTCCTTATCTC 3'	283bp	60^{0}C
p21	CDKN1A HD Ex2	F 5' TTCTACCACTCCAAACGCC 3' R 5' GAAGATGTAGAGCGGGCCT 3'	412bp	56^{0}C

PCR-based Methylation Specific Restriction Digestion (MSRA) Analysis:

Approximately 100ng of paired DNA (tumor tissue and PBL) samples were digested overnight with methylation sensitive restriction enzyme, HpaII (CCGG) and HhaI (GCGC)) and methylation insensitive enzyme, MspI (CCGG), separately. *β-3A adaptin* gene (K1) and *RARβ2* exon-1 (K2) were used as DNA digestion control and DNA integrity control, respectively.[41] Details of the primers used in this analysis were tabulated in table 2.

Table 2: Details of primers used in MSRA.

Gene	Primer	Amplicon size (bp)	Tm (^{0}C)
SH3GL2	F 5' GAGTGTTTCTCCGCAAGAGC 3' R 5' TGAGTGGCTTTATGGAACTGC 3'	217bp	52^{0}C
p14	F 5' GCCTGCGGGGCGGAGAT 3' R 5' GCGGCTGCTGCCCTAGA 3'	316bp	62^{0}C
p16	F 5' GAAGAAAGAGGAGGGGCTG 3' R 5' GCGCTACCTGATTCCAATTC 3'	340bp	56^{0}C
p15	F 5' CGCACCCTGCGGCCAGA 3' R 5' AGTGGCCGAGCGGCCGG 3'	137bp	66^{0}C
FANCC	F 5' TTTTACCCCGTTGACAAAGC 3' R 5' CGGTACTGCTCCAGTGTTCC 3'	175bp	62^{0}C
PTCH1	F 5' CGAGGAGCACAAGAAAGCAG 3' R 5' AGAAAGAGCCAGCGAATCC 3'	295bp	50^{0}C
PHF2	F 5' GTGGTGTCCCAACCAGAAAC 3' R 5' GGGACCCCGAGGATAAGATA 3'	223bp	58^{0}C
p21	F 5’ GGGGCTCATTCTAACAGTGC 3’ R 5’ GCGCGGCCCTGATATACAA 3’	334bp	58^{0}C

mRNA expression analysis by quantitative real-time PCR (qRT-PCR):

mRNA expression analysis of the genes were performed by quantitative reverse transcriptase PCR (qRT-PCR) in 38 tumor samples; 12 normal urothelium collected as bladder tissue adjacent to tumor were also included. β2-microglobulin (B2M) was used as internal control.[42] Primer sequences used in the analysis were given in table 3. The threshold cycle (C_T) of gene of interest was normalized by the C_T of internal control to calculate the dC_T value. The mean dCT value of 12 normal urothelium was taken as calibrator to calculate the ddC_T value of each tumor sample; finally relative fold change of gene expression in each sample was calculated by following formula according to Livak et al.[43]

2^{-DDCT} = [(C_T gene of interest - C_T internal control) sample A - (C_T gene of interest - C_T internal control) sample B]. A relative fold change value of ≤0.66 was considered as reduced expression and ≥1.5 as overexpression.[44]

Table 3: Details of primers used in qRT-PCR.

Gene	Primer	Location	Amplicon size (bp)	Tm (^{0}C)
SH3GL2	F 5' CACGAATGAGCCTGG 3' R 5' GGCAACCAGAATTTCCACAT 3'	Exon 8-9	272bp	50^{0}C
p14	F 5' CCCTCGTGCTGATGCTACTGA 3' R 5' ACCACCAGCGTGTCCAGGAA 3'	Exon 1-2	200bp	55^{0}C
p16	F 5' GCTGCCCAACGCACCGAATA 3' R 5' ACCACCAGCGTGTCCAGGAA 3'	Exon 1-2	180bp	55^{0}C
p15	F 5' CGGGGACTAGTGGAGAAGGT 3' R 5' ACCAGCGTGTCCAGGAAG 3'	Exon 1-2	221bp	55^{0}C
FANCC	F 5' TGGAGGCTCTCCTCATCTGT 3' R 5' GCATTCGATCCTTCTCAGACA 3'	Exon 7-8	225bp	58^{0}C
PTCH1	F 5' GACCGGGACTATCTGCA 3' R 5' GAGGAGGCCCACAACC 3'	Exon 1-2	186bp	59^{0}C
PHF2	F 5' ACTCCTGCCTGCAGACCAC 3' R 5' TCGACCGGGACTTAAAGATG 3'	Exon 18-19	258bp	55^{0}C
β2-microglobulin	F 5' GTGCTCGCGCTACTCTCTCT 3' R 5' TCAATGTCGGATGGATGAAA 3'	Exon 1-2	143bp	50^{0}C
p21	F 5'GTCTTGTACCCTTGTGCCT 3' F 5'GAAGATGTAGAGCGGGCCT 3'	Exon 2-3	215bp	56^{0}C
p53	F 5' GCCCCTCCTCAGCATCTTAT 3' R 5' GTACAGTCAGAGCCAACCTC 3'	Exon 6-7	125bp	63^{0}C
EGFR	F 5' CCAGACAACTGTATCCAGTG 3' R 5' GTTGGACAGCCTTCAAGAC 3'	Exon 15-16	188bp	58^{0}C

Cell line and 5-aza-deoxycytidine (5-aza-dC) treatment:

T24 bladder cancer cell line was cultured following standard method. For cytotoxicity assessment of 5-azadC treatment, sulforhodamine B (SRB) assay were performed. Finally, optical density was determined at 510 nm in a microplate reader.[45] After determining the IC50 dose by SRB assay, T24 cells were treated with IC30 (0.5 μM), IC50 (1.64 μM) and IC70 (2.5 μM) doses of 5-aza-dC drug with a simultaneous culture of cells without 5-aza-dC as control.

Expression analysis by Immunochemistry:

Immunohistochemical analysis was done according to standard staining protocol[46] using 3-5µm sections of paraffin embedded tissue and recommended dilution of primary antibodies (table 4). Slides were developed using 3,3'-diaminobenzidiene as chromogen. Evaluation of protein expression (low, intermediate, high) was conducted by two unbiased individuals as described by Perrone et al.[47] Slides were imaged with Leica microscope (Leica DMi8 automated, Wetzlar, Germany).

Table 4: Details of antibodies used.

Name of antibody	Origin	Dilution
Anti-Ki67	Rabbit	1:100
Anti- PHF2	Rabbit	1:100
Anti- P53	Rabbit	1:100
Anti- P21	Rabbit	1:100
Anti- SH3GL2	Rabbit	1:100
Anti- EGFR	Rabbit	1:100
Goat anti-rabbit IgG-HRP	Goat	1:500

Statistical analysis:

Level of significance (p-value <0.05) were analysed by Fisher's exact test and t-test. Spearman correlation were used to assess correlation coefficient between two groups. Heat-maps of deletion were generated by using software from Morpheus Broad Institute (https://software.broadinstitute.org/morpheus/). Bladder cancer patients were followed up for a maximum period of 5 years for the survival analysis according to Kaplan-Meier model and Log rank test. Cox proportional regression model were used to assess the hazard of different prognostic factors. The software of SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad PRISM 8.0.1 (244) (GraphPad Software, LaJolla, California, USA) were used for statistical analysis.

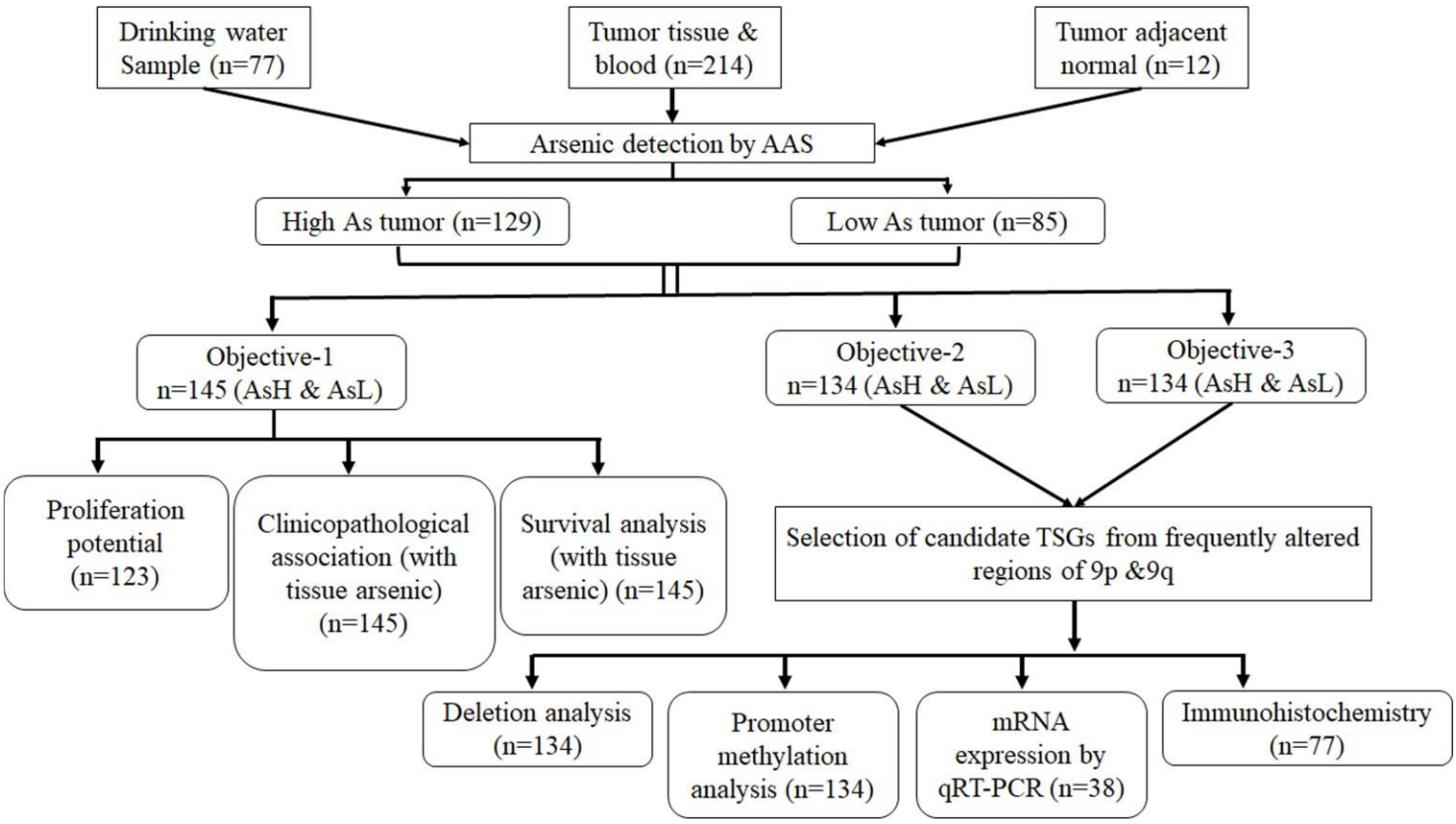

Drinking water Sample (n=77)
Tumor tissue & blood (n=214)
Tumor adjacent normal (n=12)
Arsenic detection by AAS
High As tumor (n=129)
Low As tumor (n=85)
Objective-1 n=145 (AsH & AsL)
Objective-2 n=134 (AsH & AsL)
Objective-3 n=134 (AsH & AsL)
Proliferation potential (n=123)
Clinicopathological association (with tissue arsenic) (n=145)
Survival analysis (with tissue arsenic) (n=145)
Selection of candidate TSGs from frequently altered regions of 9p &9q
Deletion analysis (n=134)
Promoter methylation analysis (n=134)
mRNA expression by qRT-PCR (n=38)
Immunohistochemistry (n=77)

Figure 1: Summary of total study plan. AAS, Atomic Absorption Spectroscopy. As, Arsenic. AsL, low level of tissue arsenic. AsH, high level of tissue arsenic.

Results:

Objective 1: Analysis of key etiological factors with special reference to arsenic associated with development of bladder cancer in West Bengal.

1A. Characteristics of study participants:

Participants of this study were mainly from districts adjacent to the Gangetic belt of West Bengal (Figure 2a). Sociodemographic data of studied participants are presented in Table 5.

Table 5: Sociodemographic and clinicopathological characteristics of patients included in the study.

Characteristics			Number (%)
Age		Median age (range)	65 (32-86)
Sex		Male	126 (87%)
		Female	19 (13%)
Arsenic status of ground water		Arsenic affected	97 (67%)
		Arsenic safe	48 (33%)
Source of drinking water		Underground	118 (81%)
		Surface	27 (19%)
Smoking history	Smoker	PY, 1-10 (n=33)	86 (59%)
		PY, 11-20 (n=15)	
		PY, >20 (n=38)	
	Non-smoker		59 (41%)
Alcohol consumption		Yes	19 (13%)
		No	126 (87%)
Occupational exposure to As/ Cd		Yes	10 (7%)
		No	135 (93%)
Family history of cancer		Yes	7 (5%)
		No	138 (95%)
Diabetic history		Yes	13 (9%)
		No	132 (91%)
Pathological stage		Ta[#]	29 (20%)
		T1[#]	51 (35%)
		T2*****	46 (32%)
		T3*****+T4*	19 (13%)
Histologic grade		High	87 (60%)
		Low	58 (40%)
Nodal invasion or metastasis		Yes	14 (10%)
		No	131 (90%)

PY, pack year of tobacco smoking; Surgical treatment employed: [#]TURBT (Transurethral Resection of bladder tumor), *radical cystectomy.

Comparatively higher frequency of cases were documented in arsenic affected blocks/wards than in arsenic safe ones in each district. Overall distribution of cases followed similar trend (Figure 2b&c). Among 145 malignant cases 55% (80/145) were non-muscle-invasive bladder cancer (NMIBC); the rest 45% (65/145) were muscle-invasive bladder cancer (MIBC), that could be further classified as Ta (n=29), T1 (n=51), T2 (n=46) and T4 + T4 (n=19). 11 cases of nodal invasion and 3 of distant metastasis were recorded. (Table 5).

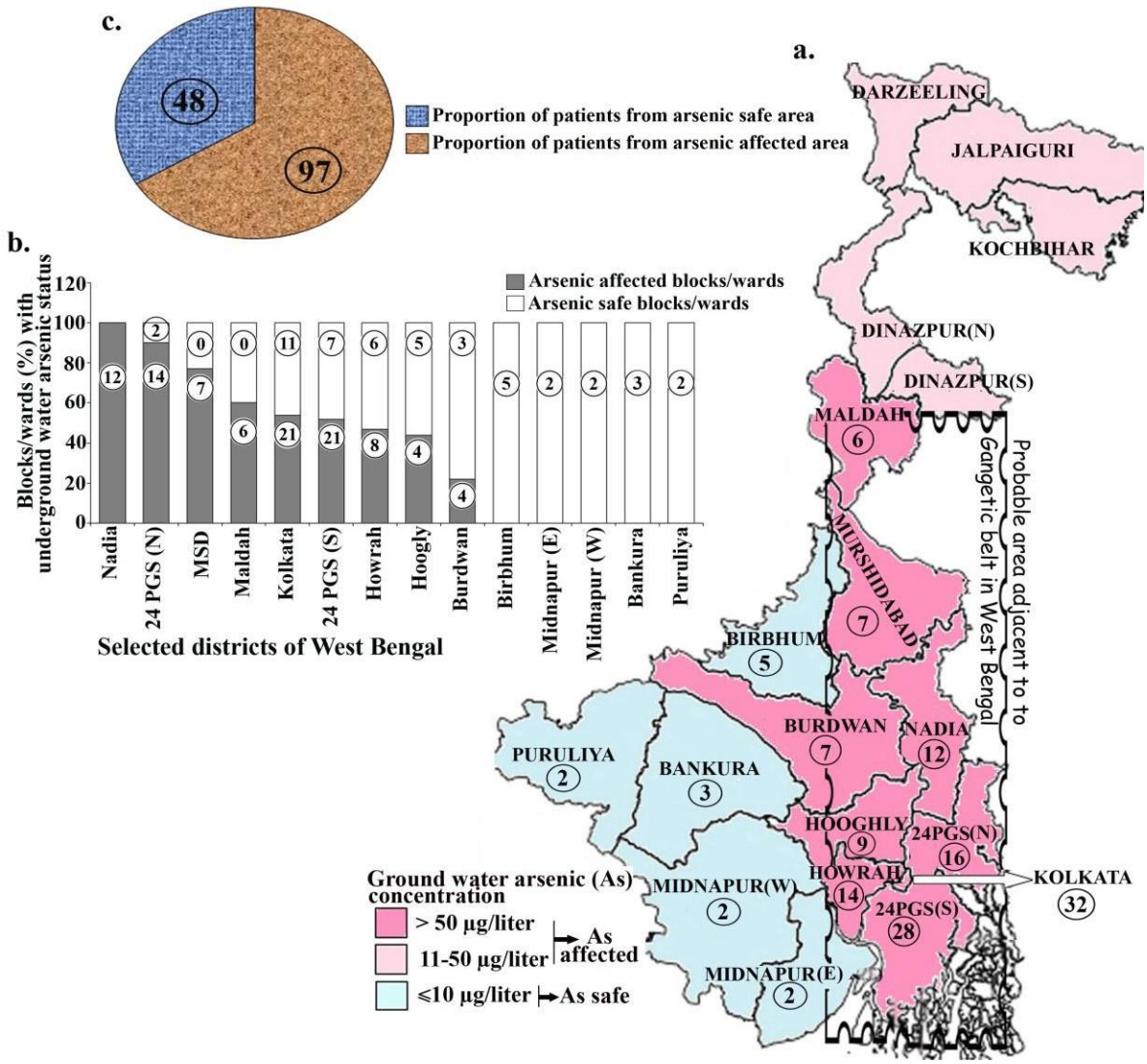


Figure 2: Distribution of bladder cancer patients included in the study. (a) Distribution of patients in different districts of West Bengal. (b) Distribution in arsenic affected and safe blocks/wards in each districts wherefrom patients are reported. (c) Overall distribution of patients in arsenic affected and safe areas. Numbers inside circle indicates number of patients.

1B. Total arsenic in bladder tumor tissue & drinking water:

Detection of arsenic (As) concentration in bladder tumor showed significant differences in patients from arsenic affected area and that from safe area (median: 256 µg/kg vs 77 µg/kg; $p < 0.0001$) (Figure 3a). 77 drinking water samples collected from the patients during their follow-up and measured for total arsenic which showed significantly positive correlation ($r = 0.65$; $p < 0.0001$) with respective tumor tissue arsenic content (Figure 3b). As per the recommendation of US Environmental Protection Agency, 1993[48,49], 10–100 ppb (µg/kg) might be the range of As level in soft tissue of unexposed individuals. In our study, bladder tumors of As concentration ≤100ppb were categorized as tumors with low arsenic (AsL) and those with

>100ppb as tumors with high arsenic (AsH) accordingly. Among cases in which both tissue and water arsenic data were available, AsH tumors were significantly more frequent ($p < 0.0001$) in patients using contaminated drinking water (>10 µg/l) than those having access to safe one (≤10 µg/l) (Figure 3c). Furthermore, high levels of arsenic was found to be accumulated in tumors of patients from arsenic affected blocks/wards compared to those from arsenic safe areas (Figure 3d).

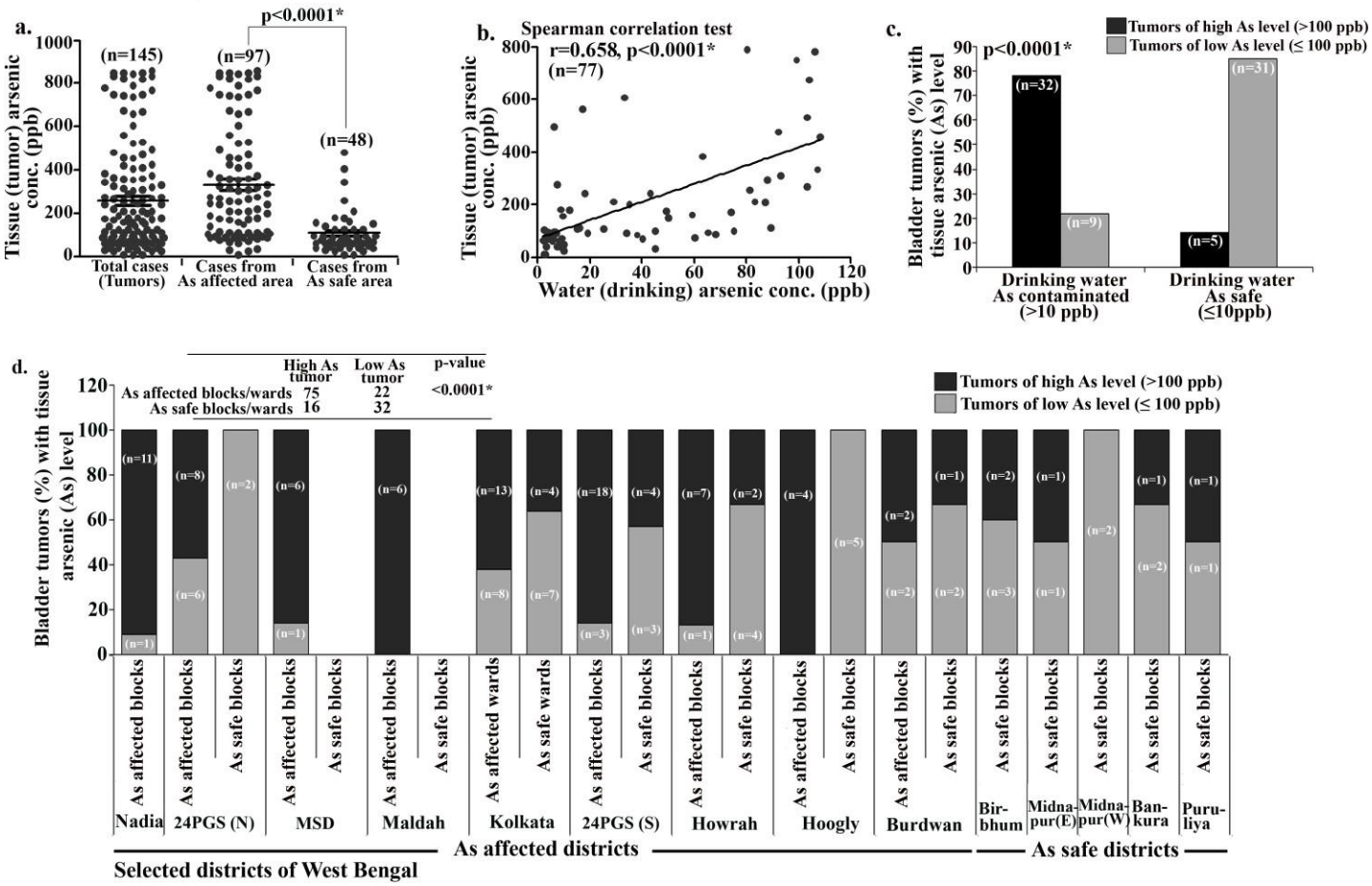


Figure 3: Association of tumor tissue arsenic with level of arsenic in drinking water as well as available records of underground water of patients' locality. (a) Total As concentration in tumors and comparison of the same in patients from arsenic affected and safe areas. (b) Correlation between As concentration of tumors and respective drinking water samples of patients. (c) Association of tumors with high and low As level with arsenic concentration of drinking water samples of patients. (d) Association of tumor tissue arsenic with reported groundwater arsenic status of respective districts.

1C. Ki67 expression in bladder tumor:

Expression of Ki67 at protein level was assessed by immunohistochemistry to check proliferation potential of bladder tumor. Ki67 was detected in nucleus of basal and parabasal cells of normal urothelium and in tumor more frequent expression was evident. (Figure 4a).

20% positivity of Ki67 had been set as the upper cut-off in bladder cancer, and any score above this value was reported to be an adverse disease outcome predictor.[50] Total As concentration of the samples showing overexpression of Ki67 (57%; 70/123) was significantly higher (p=0.0010) compared to those samples expressing normal level of protein in the nucleus (43%; 53/123) (Figure 4b). Association of Ki67-overexpression with tissue arsenic was significant (p = 0.0002) in MIBC samples, but not in NMIBC (p = 0.61) (Figure 4c).

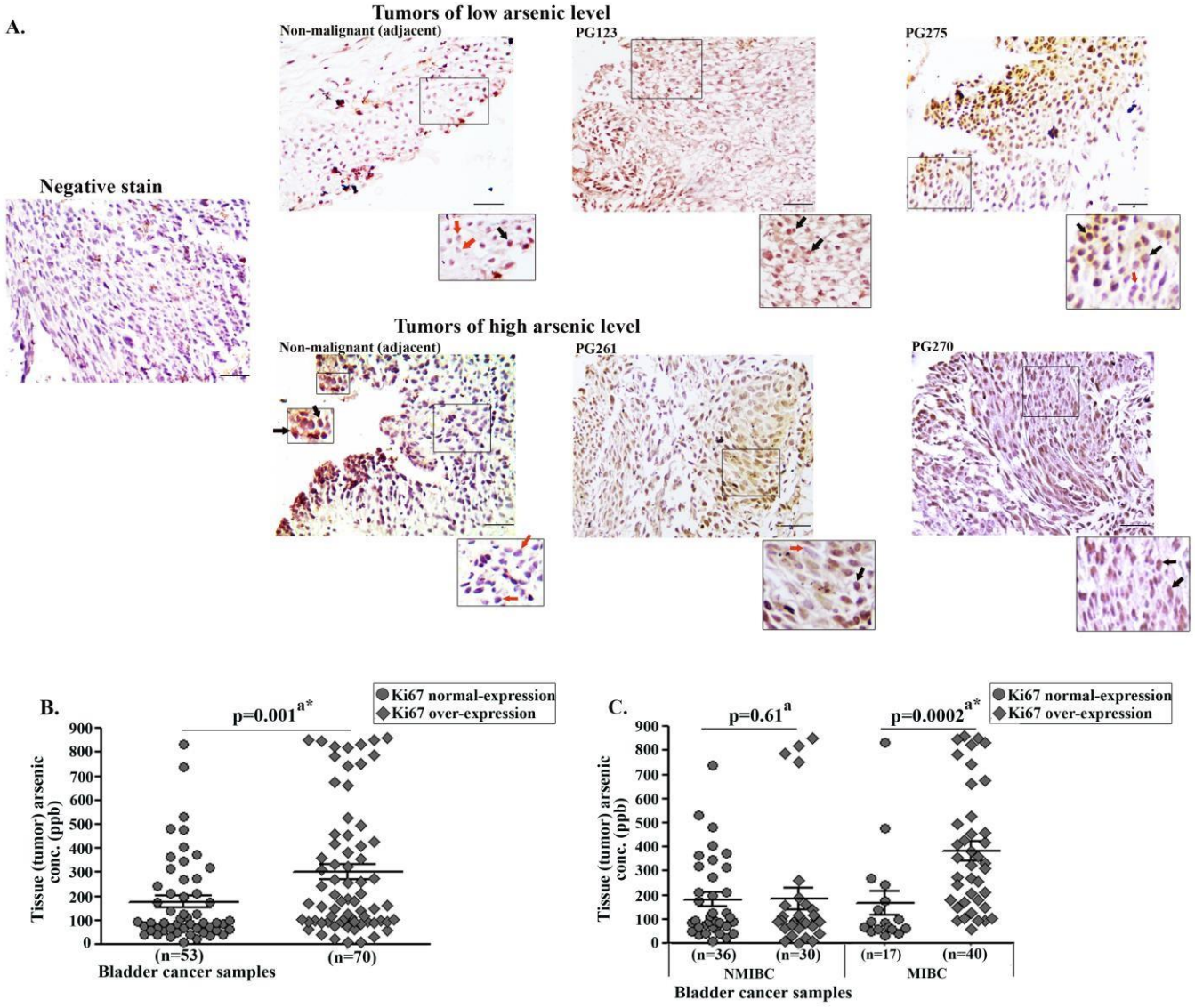


Figure 4: Protein expression of Ki67 in bladder tumor samples. (A) Representative immunohistological staining pattern of Ki67 protein in adjacent normal bladder urothelium, tumor tissues with low and high level of arsenic, along with negative stain. Black & red arrow-positive & negative expression of the protein. (B) Distribution of As concentration of tumors against their Ki67 expression level. (C) Association of tumor tissue arsenic with Ki67 expression level in NMIBC and MIBC samples.

1D. Clinicopathological association:

Arsenic concentration in bladder tumor significantly increased with advancement of pathological stages (Ta vs T1 vs T2 vs T3 + T4; p = 0.001; NMIBC vs MIBC; p = 0.0009) (Figure 5Aa & 5Ab). Tumor stages did not show any significant changes among tobacco smokers and nonsmokers (T- vs T+) ((Figure 5Ba) and between tobacco users stratified according to pack years of smoking and tumor stages grouped as NMIBC (Ta + T1) and MIBC (T2 + T3 + T4) types (data not shown). Both, tumor arsenic concentration and tobacco smoking did not differ significantly with histologic grades of tumor (data not shown). Furthermore, tumors were categorized according to arsenic concentration (arsenic high/AsH: >100 ppb and arsenic low/AsL: ≤100 ppb) and combined with tobacco smoking status. Frequency of MIBC cases increased gradually in groups positive for one criteria to positive for both the criteria (T+, As-L< T-, As-H< T+, As-H) compared to those negative for both the criteria (T-, As-L) (Figure 5Bb). Distribution of MIBC showed an increasing trend with increase in level of tissue arsenic (T+, As-L vs T-, As-H; p = 0.242 and T+, As-L vs T+, As-H; p = 0.122) and remained comparable among high arsenic level tumors (T-, As-H vs T+, As-H; p = 0.834) irrespective of the tobacco smoking habit (Figure 5Bb).

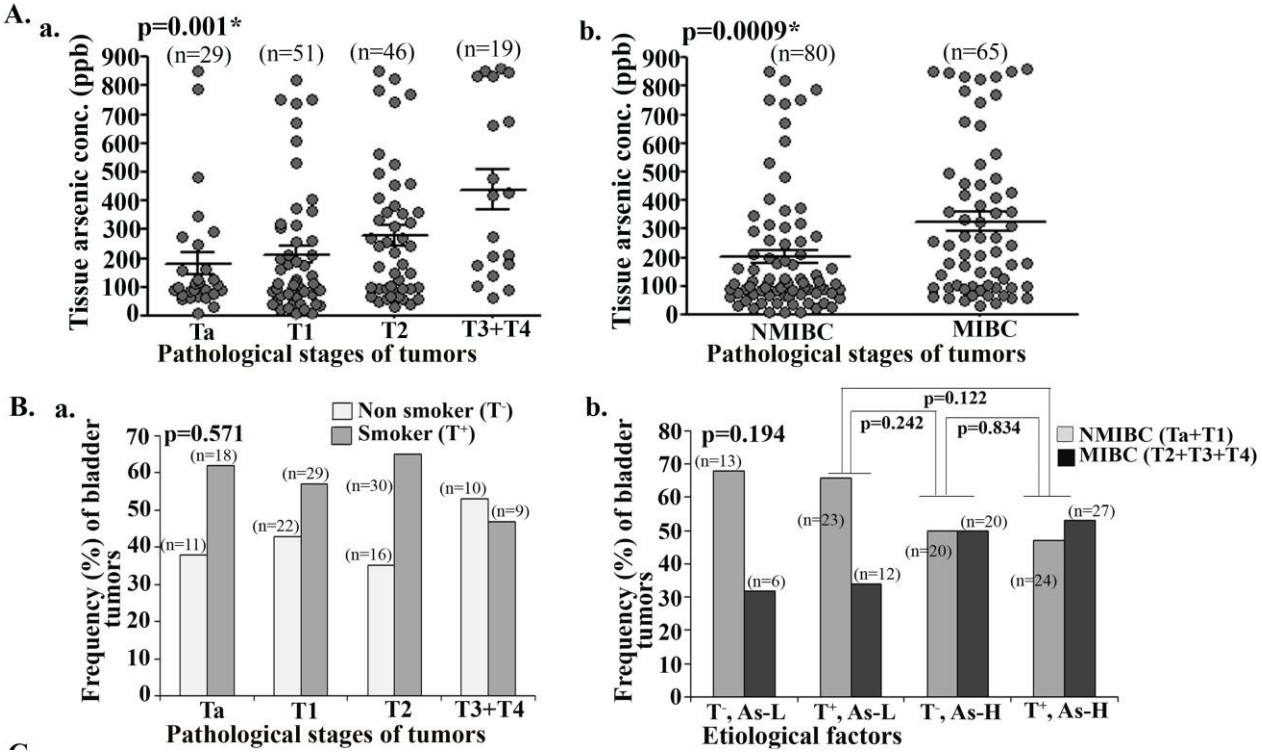


Figure 5: Clinicopathological analysis of tumor samples included in the study. **A.** Comparison of arsenic content of tumor (a) with pathological stages & (b) in NMIBC and MIBC categories.

B. Association of (a) tobacco smoking habit with pathological stages of tumor & (b) combined status of arsenic and tobacco with tumor invasiveness (NMIBC vs MIBC).

1E. Patient survival:

Median of follow-up time of the patients were 27 months. 2 patients died from NMIBC group; in 18 cases, the disease recurred, of which 12 were progressed to MIBC tumors. 7 individuals died from MIBC group while 5 reported for cancer-specific death, and 29 cases of recurrence was found. With respect to overall survival (OS), significantly poor prognosis ($p < 0.0001$) was observed in patients with high level of tumor tissue arsenic (Figure 6A). However, in Kaplan-Meier analysis, the OS did not differ significantly among tobacco smokers and nonsmokers (Figure 6B). When two etiological factors were combined, the worst prognosis was recorded in tobacco smokers having high arsenic level in tumors (As+, T+) and the best prognosis in those negative for both factors (As- T-); the survival status of nonsmokers having high tumor arsenic level (As+, T-) and of nonsmokers having low tumor arsenic (As- T-) was found to be comparable to that of As+, T+ and As- T- groups, respectively (Figure 6C).

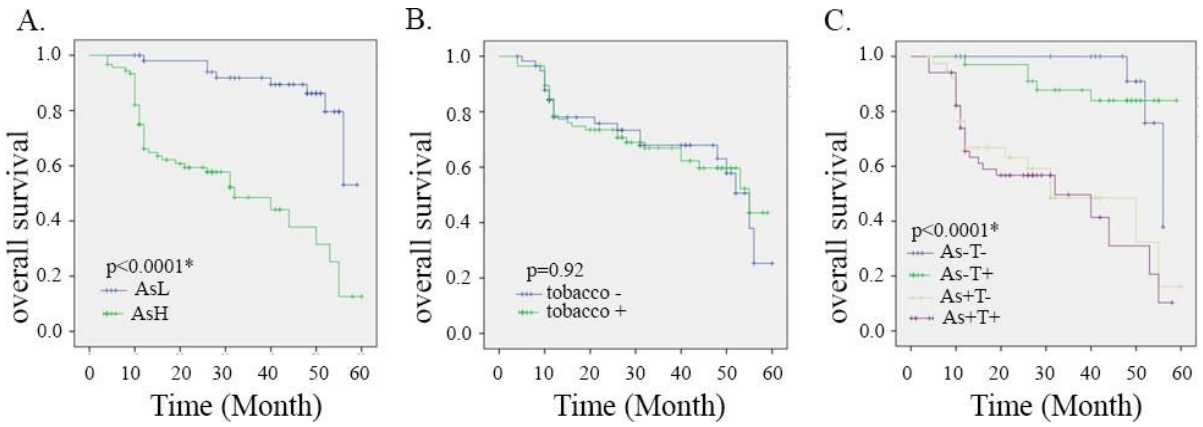


Figure 6: Survival analysis of the studied patients. Kaplan-Meier Survival plot of patients with (A) tissue arsenic (AsL vs AsH. (B) Smoking habit of tobacco (tobacco- vs tobacco+) and (C) both arsenic and smoking habit (As-T-, As-T+, As+T-, and As+T+).

Univariate Cox regression analysis showed significant association of high tissue arsenic level, tumor stage, grade and Ki67 overexpression for poor OS (HR: 6.60, 95% CI: 3.03-14.3; $p < 0.0001$, HR: 4.52, 95% CI: 2.45-8.31; $p < 0.0001$, HR: 2.08, 95% CI: 1.12-3.86; $p=0.019$ and HR: 4.87; 95% CI: 2.35-10.05; $p < 0.0001$, respectively). Multivariate analysis of all the factors were able to persist the trend for tissue arsenic, tumor stage and Ki67 overexpression (HR: 5.73, 95% CI: 2.42-13.51; $p<0.0001$, HR: 2.35, 95% CI: 1.22-4.52; $p= 0.01$, and HR: 2.75, 95% CI: 1.27-5.91; $p = 0.01$, respectively) (Table 6).

Table 6. Cox regression analysis for association of different parameters with overall survival of patients

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
Tissue (bladder tumor) arsenic level£ [High vs. Low]	6.60 (3.03-14.3)	<0.0001*	5.73 (2.42-13.51)	<0.0001*
Tumor stage# [NMIBC vs MIBC]	4.52 (2.45-8.31)	<0.0001*	2.35 (1.22-4.52)	0.010*
Tumor Grade€ [High vs. Low]	2.08 (1.12-3.86)	0.019*		
Ki67 expression± [Over-expression vs. Normal]	4.87 (2.35-10.05)	<0.0001*	2.75 (1.27-5.91)	0.010*
Smoking [Yes vs. No]	0.97 (0.55-1.69)	0.92		
Alcohol [Yes vs. No]	1.06 (0.47-2.36)	0.88		

£ High: >100 µg/kg; Low: ≤100 µg/kg
according to 2010 American Joint Committee on Cancer manual
€ according to the 2004 WHO classification system.
± Over-expression: >20% positivity; Normal: <20% positivity
$progression from non-muscle invasive to muscle invasive stages
HR, hazard ratio; CI, confidence interval. * p value significant.

Objective 2: High resolution copy number variation analysis (CNV) for identification of frequently altered (deletion/amplification) regions of chromosome 9p and 9q associated with development of urinary bladder cancer.

&

Objective 3: Promoter methylation followed by expression analysis of candidate genes located in the frequently altered chromosomal regions.

2A. Identification of significantly altered regions of chromosome 9:

Our research group has done high resolution CGH-SNP microarray analysis of 10 bladder (AsH, n=5 and AsL, n=5) cancer samples and deposited the data to GEO database (acceptance

number GSE122514). We have extracted the data to identify chromosomal regions of 9p & 9q showing significantly high frequency of copy number variation in AsH samples compared to AsL. Based upon the trend of our data, 40% (2/5) alteration frequency is taken as a cut-off to define a chromosomal region to be deleted.[50] On the basis of this criteria, the deletion status of cytobands 9p24.3-24.2, 9p24.1, 9p22, 9p21.3 from chromosome 9p and cytobands 9q21.11-21.13, 9q21.31-21.33, 9q22.31-22.33, 9q31.1-31.3, 9q32, 9q33.1-33.2 from 9q were considered deleted in AsH samples compared to AsL ones.

Of deleted chromosomal regions, we have selected **9p21-22** from short arm (p arm) and **9q22.3** from long arm (q arm) of chromosome 9 for more detailed analysis due to previous reports on association of the deleted regions with BLCA and presence of important TSGs within the deleted regions having contribution in tumorigenesis.[51,22,23,24]

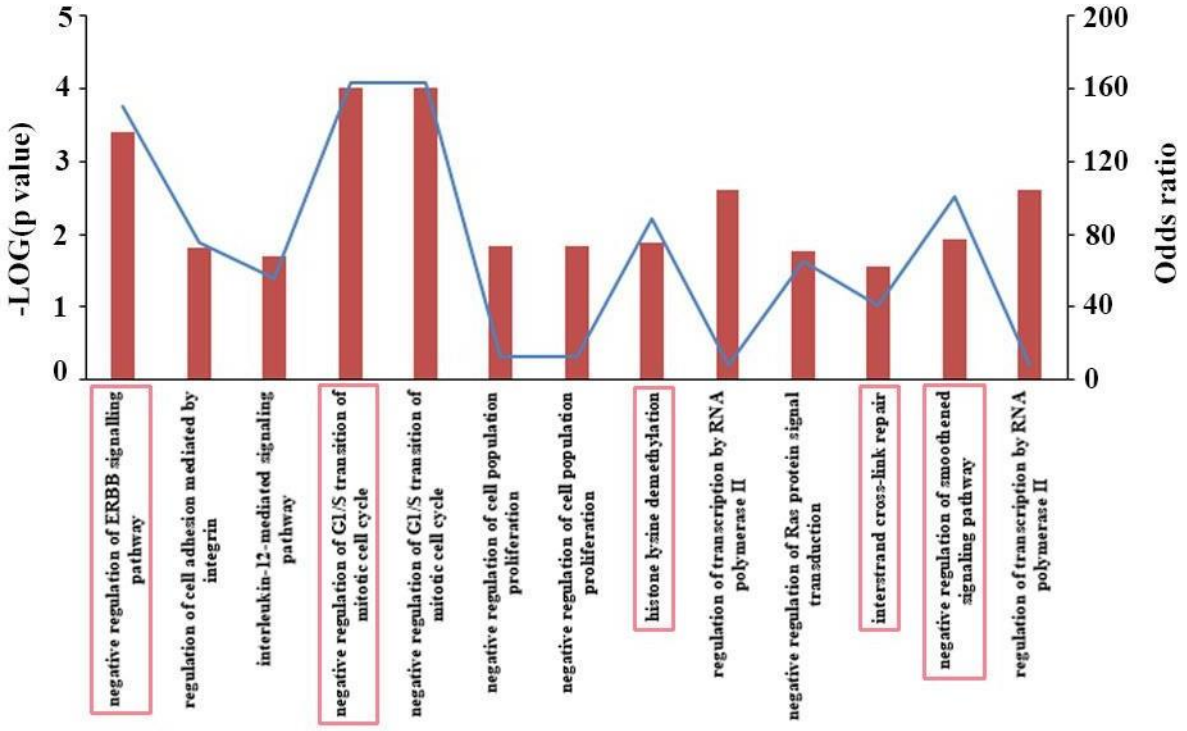
2B. Genes present in the deleted chromosomal regions (9p21-22 & 9q22.3) and associated molecular pathways:

The genes of the deleted chromosomal regions having a report or potential TSG like function were selected (Table 7). To identify the molecular pathways that seem to be dysregulated during arsenic- induced bladder carcinogenesis, the selected genes of deleted chromosomal regions were then subjected to the Gene Ontology (GO) enrichment analysis using the Enrichr web-tool.[52]

Table 7. List of genes selected for pathway enrichment analysis

Chr 9p21-22			Chr 9q22.3		
Gene	Alteration frequency		Gene	Alteration frequency	
	AsH (n=5)	AsL (n=5)		AsH (n=5)	AsL (n=5)
BNC2	60% (3)	40% (2)	ROR2	60% (3)	20% (1)
SH3GL2	80% (4)	20% (1)	NINJ1	40% (2)	0% (0)
KIAA1797	100% (5)	80% (4)	WNK2	80% (4)	40% (2)
PTPLAD2	100% (5)	40% (2)	PHF2	60% (3)	20% (1)
ACER2	60% (3)	40% (2)	BARX1	60% (3)	20% (1)
MTAP	80% (4)	80% (4)	FBP1	60% (3)	40% (2)
CDKN2A	40% (2)	0% (0)	FANCC	60% (3)	20% (1)
CDKN2B	80% (4)	80% (4)	PTCH1	40% (2)	20% (1)
			C9orf102	60% (3)	20% (1)
			ZNF367	60% (3)	20% (1)

Among significantly altered pathways, "negative regulation of ERBB signalling" and "negative regulation of G1/S transition" constituting genes of chromosomal 9p21-22 region and, "histone lysine demethylation", "inter-strand crosslink repair" and "negative regulation of smoothened signalling" constituting the genes of chromosomal 9p22.3 region were selected (Figure 7) for validation in independent sample sets (AsH & AsL), considering the importance of the same (pathways) in tumorigenesis process.[53,54,55,56,57,58,59]



Genes selected	Chromosomal location	Frequency of deletion in AsH samples (%)	Associated pathway
SH3GL2	9p22.2	80	negative regulation of ERBB signalling
CDKN2A	9p21.3	40	negative regulation of G1/S transition
CDKN2B	9p21.3	80	negative regulation of G1/S transition
PHF2	9q22.31	60	histone lysine demethylation
FANCC	9q22.32	60	interstrand crosslink repair
PTCH1	9q22.32	40	negative regulation of smoothened signalling

Figure 7: Graphical overview of relevant significant pathways after enrichment analysis of selected gene sets from 9p21-22 & 9q22.3 along with final list of pathways for validation.

2C. Validation of selected molecular pathways by high resolution copy number variation analysis followed by promoter methylation and expression analyses of constituent candidate TSGs:

For validation purpose, 134 primary bladder tumor samples were recruited. After arsenic detection, 75 tumors were considered under AsH and 59 tumors were considered under AsL catrgories. The demographic and histopathological characteristics of tumor samples were described in Table 8.

Table 8: Demographic & histopathological details of studied participants.

Characteristics			**Number (%)**
Age		Median age (range)	63 (32-86)
Sex		Male	113 (84%)
		Female	21 (16%)
Arsenic status of ground water		Arsenic affected	83 (62%)
		Arsenic safe	51 (38%)
Source of drinking water		Underground	112 (84%)
		Surface	22 (16%)
Tumor tissue arsenic		AsL (≤100 ppb)	59 (44%)
		AsH (>100 ppb)	75 (56%)
Smoking history	Smoker	PY, 1-10 (n=43)	79 (59%)
		PY, 11-20 (n=14)	
		PY, >20 (n=22)	
	Non-smoker		55 (41%)
Alcohol consumption		Yes	18 (13%)
		No	116 (87%)
Occupational exposure to As/ Cd		Yes	9 (7%)
		No	125 (93%)
Family history of cancer		Yes	2 (4%)
		No	55 (96%)
Diabetic history		Yes	11 (8%)
		No	123 (92%)
Pathological stage		Ta[#]	34 (25%)
		T1[#]	53 (40%)
		T2*****	35 (26%)
		T3*****+T4*	12 (9%)
Histologic grade		High	70 (52%)
		Low	64 (48%)
Nodal invasion or metastasis		Yes	15(11%)
		No	119(89%)

PY, pack year of tobacco smoking; Surgical treatment employed: [#]TURBT (Transurethral Resection of bladder tumor), *radical cystectomy.

(1) Negative regulation of G1/S transition of mitotic cell cycle pathway

Genes involved: *CDKN2A, CDKN2B*

Chromosome 9p21 harbours two adjacent gene loci namely *INK4/ARF and CDKN2B;* the former encodes two bonafide TSGs ($p14^{ARF}$ and $p16^{INK4A}$), and the later encodes another TSG, $p15^{INK4B}$. $p16^{INK4A}$ and $p14^{ARF}$ share common exons 2 and 3, but have unique first exon with independent promoters (Figure 8). CDKN2B locus consist of two exons with its promoter in the upstream of first exon.

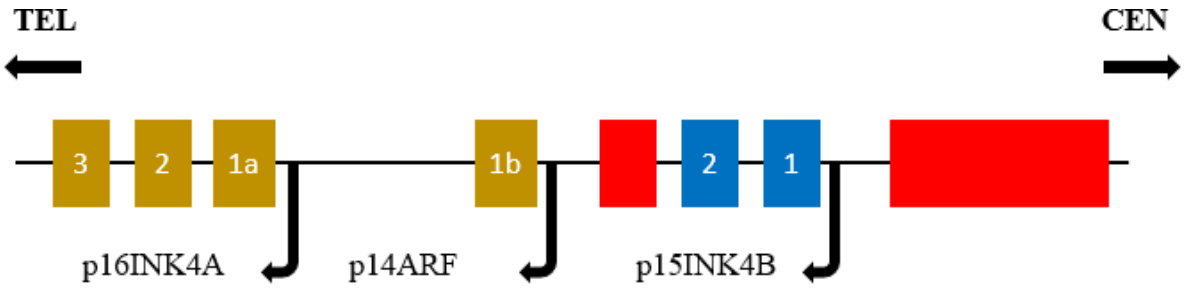


Figure 8: Details of *INK4/ARF* and *CDKN2B* locus with transcribed genes from respective promoters.

$p16^{INK4A}$ and $p15^{INK4B}$ serve as CDK inhibitors; both proteins physically interact with cdk4 and 6 to interrupt their interaction with cyclinD1, and this prevents the formation of cyclinD-cdk4/6 complex leading to the cell cycle arrest at G1-S phase. $p14^{ARF}$ binds with MDM2 that serve as a p53 ubiquitin ligase; as a result of interaction with p14arf, MDM2 is inactivated and gets sequestered within nucleolus. During tumorigenesis, both *INK4A* and *INK4B* loci were found to undergo molecular alterations leading to transcriptional inactivation or mutational defects of the constituent TSGs leading to defective cell cycle checkpoints that ultimately results abnormal cell proliferation.[60,61,62,63,64]

To explore the association of "negative regulation of G1/S transition of mitotic cell cycle pathway" in arsenic-induced bladder carcinogenesis, we analysed the deletion status *of p16, p14, p15* from whole genome CGH+SNP array data followed by its validation by CNV analysis in independent sample set. Promoter methylation of TSGs was also done on same sample set to consider arsenic-induced epigenetic alteration. Finally, genetic and epigenetic alterations were compared between AsH and AsL tumors to evaluate the functional association of the pathway in tumorigenesis under conditions of arsenic exposure.

Deletion analysis by microsatellite/ exonic markers:

Array data showed higher level of deletion (40%; 2/5) of CDKN2A locus in AsH samples compared to AsL (0%; 0/5) ones; CDKN2B locus had a comparable result between the two groups (80%; 4/5 for both AsH & AsL) (Figure 9Aa&Ba).

For validation, deletion analysis of both loci (*CDKN2A, CDKN2B*) was done by exonic markers in 75 AsH and 59 AsL samples. However, deletion frequency of both loci did not show any significant difference between AsH and AsL samples (71% vs 66%, p=0.579 for CDKN2A and 55% vs 71%, p=0.073 for CDKN2B) (Figure 9Ab&c and 9Bb&c). Additionally, deletion frequencies of CDKN2A and CDKN2B remained comparable among NMIBC and MIBC samples.

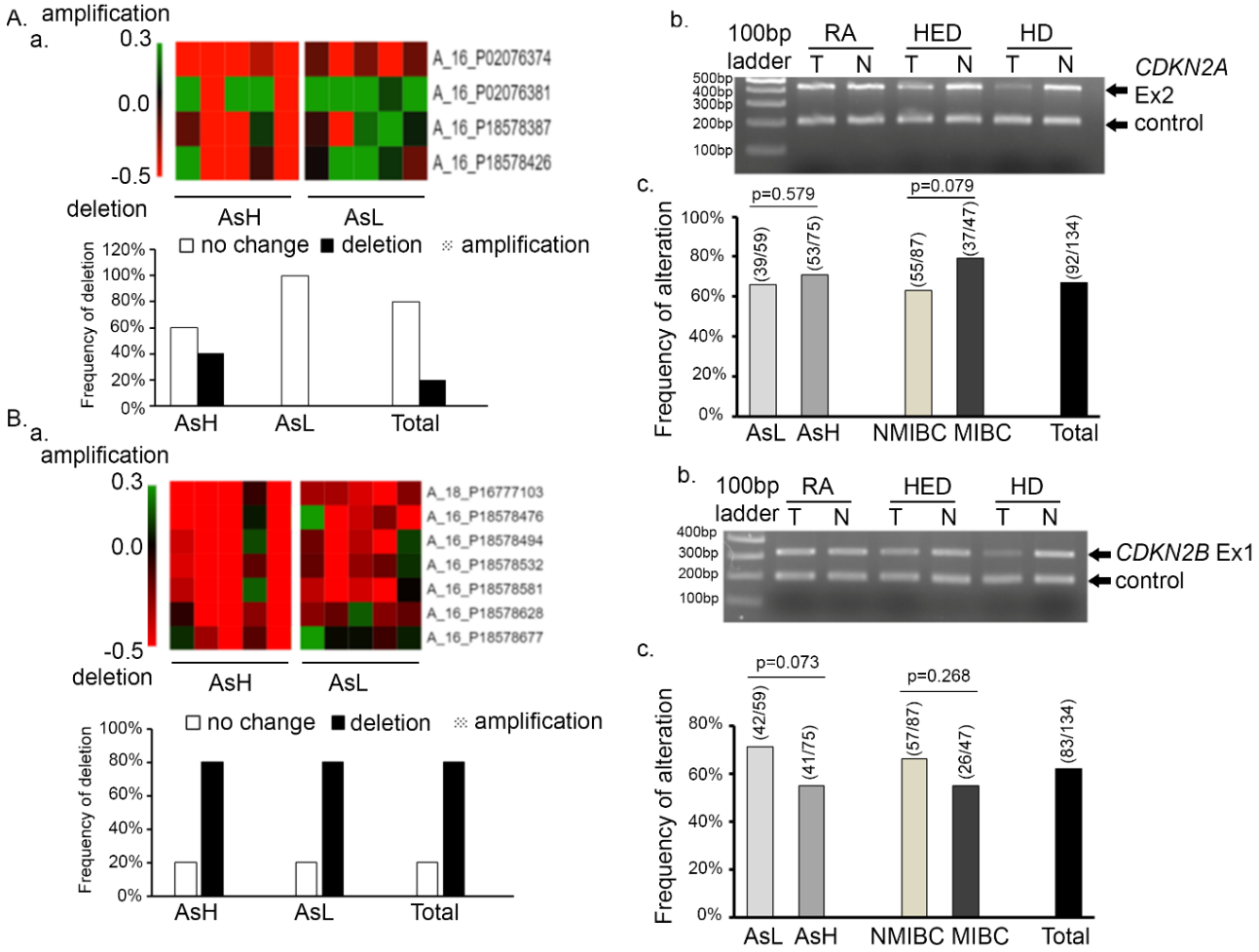


Figure 9: Deletion status of *CDKN2A* and *CDKN2B* loci. (Aa & Ba). Heat-map showing deletion status of *CDKN2A* and *CDKN2B* from CGH-SNP array data and histogram representing summarized view of the heat-map (Ab & Bb) Representative gel photograph of showing types of alteration of the genes from exonic marker-based analysis. (Ac & Bc) Histogram of frequency of deletion of *CDKN2A* and *CDKN2B*, respectively.

Promoter methylation analysis by MSRA:

CpG methylation of $p14^{ARF}$, $p16^{INK4A}$ and $p15^{INK4B}$ promoters was found in 63% (85/134), 64% (86/134), and 52% (71/134) of bladder tumor samples, respectively. But, when sample were categorized according to tumor arsenic level and pathological stages, promoter methylation frequencies of the studied gene loci remained comparable between AsH vs AsL groups (67% vs 59%, p=0.470 for $p14^{ARF}$, 63% vs 66%, p=0.719 for $p16^{INK4A}$ and 56% vs 47%, p=0.385 for $p15^{INK4B}$), and between NMIBC vs MIBC stages of tumor (Figure 10).

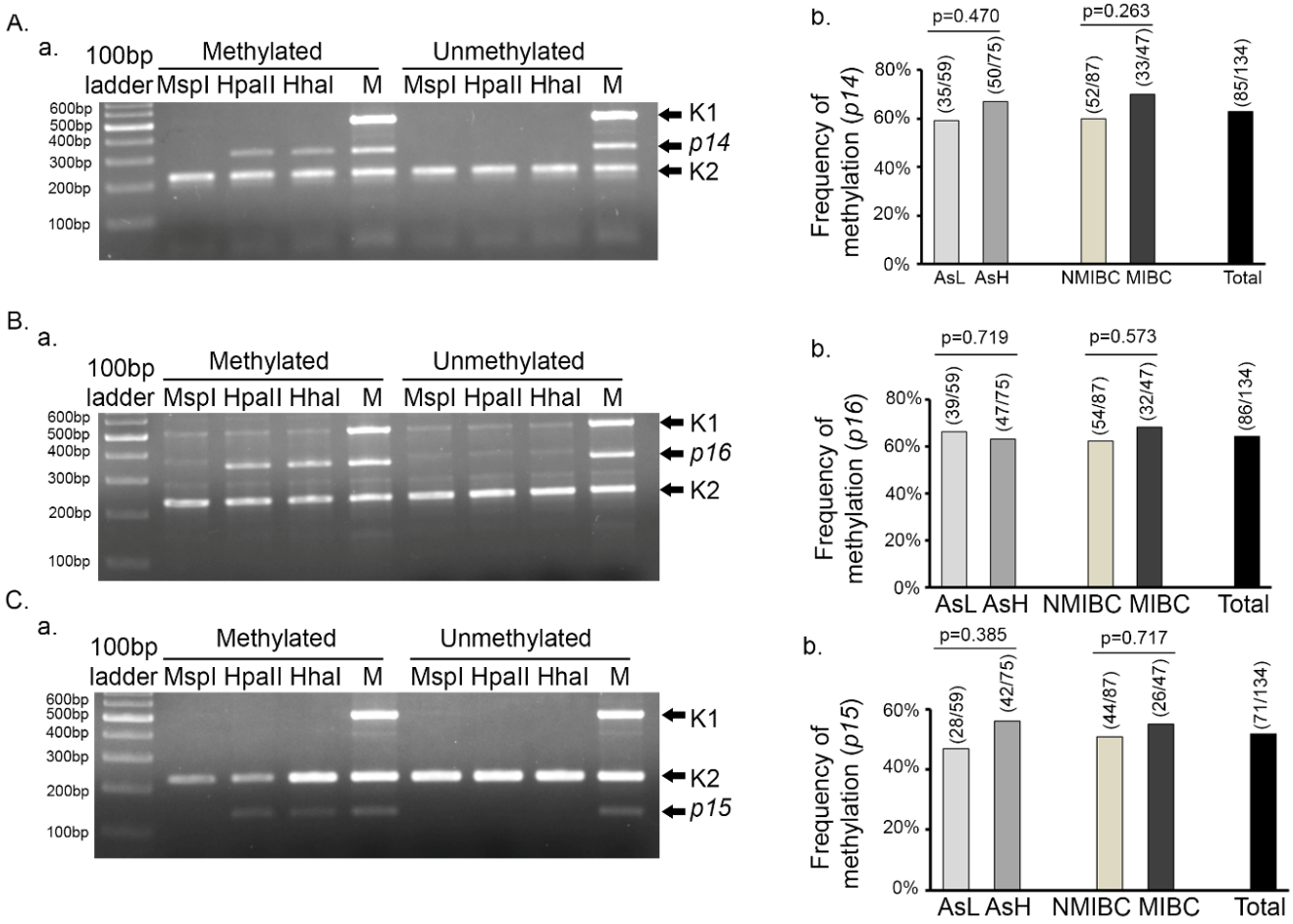


Figure 10: Methylation status of the genes. Left panel (Aa, Ba & Ca) represents the gel photograph of MSRA of *p14* (316bp), *p16* (340bp), and *p15* (137bp) where K1 (506bp) and K2 (227bp) served as digestion control and DNA integrity control; MspI and HpaII were methylation sensitive and insensitive restriction digestion enzymes, respectively. Right panel (Ab, Bb & Cb) Histogram showing methylation of frequency of the three genes.

Overall alteration of the genes:

Now, deletion and/or methylation of the studied gene loci was considered to determine the overall alteration status in each tumor sample; similar to deletion and methylation, overall alteration frequencies for $p14^{ARF}$, $p16^{INK4A}$ and $p15^{INK4B}$ did not differ significantly among AsH & AsL, and NMIBC & MIBC groups of bladder tumors (Figure 11).

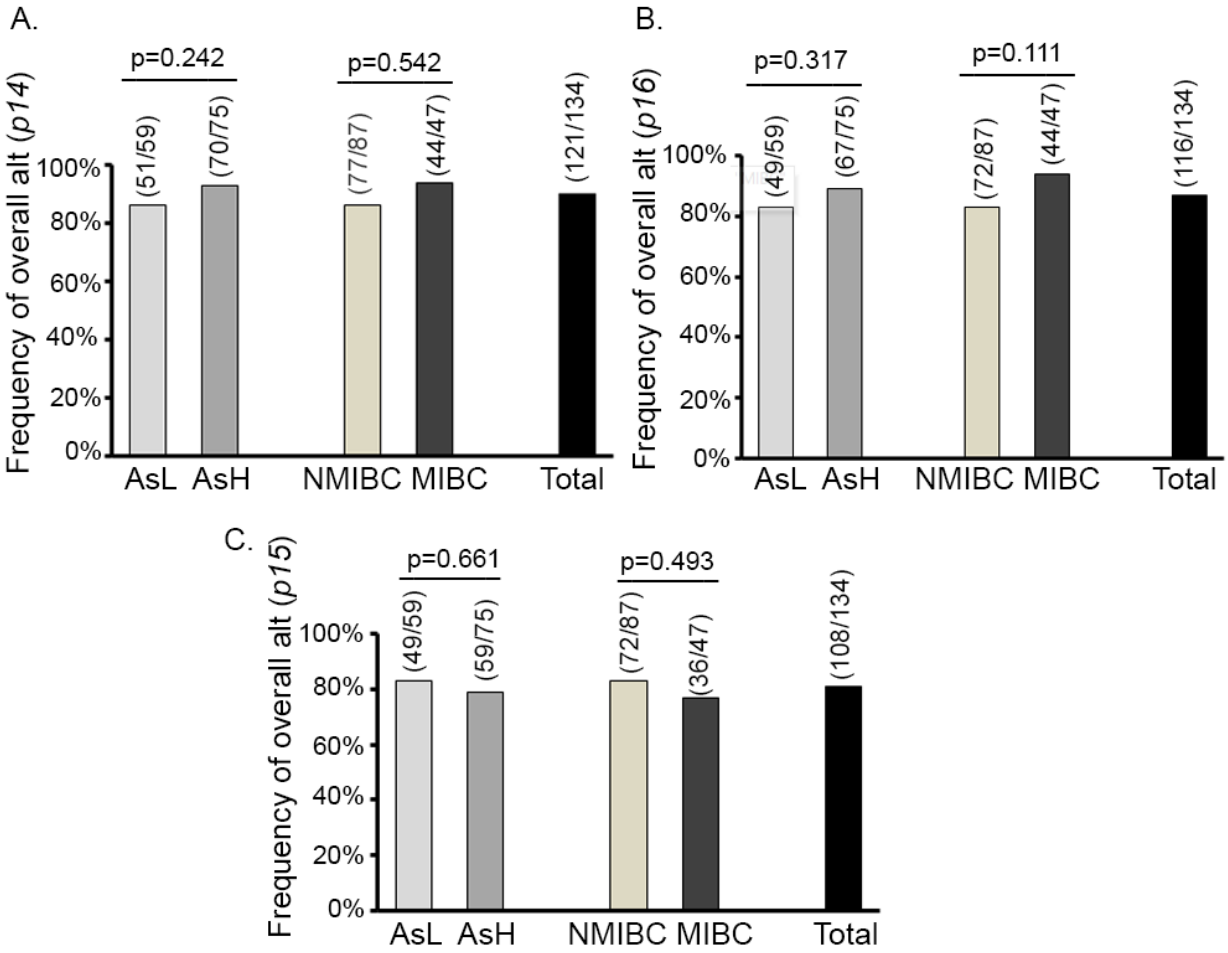


Figure 11: Overall alteration of the genes. Histogram showing overall alteration frequencies of the genes, *p14* (A), *p16* (B), and *p15* (C) between AsH-AsL and NMIBC-MIBC groups.

mRNA expression analysis:

mRNA expression analysis of *p14*, *p16*, and *p15* was done by qRT-PCR in 38 bladder tumor samples with known deletion and promoter methylation status for the gene loci; also, among 38 tumors, 20 samples belong to AsH and 18 belong to AsL categories. In our studied samples, desired amplicon of specific size was readily detected in agarose gel for all the three genes (Figure 12 Aa, Ba & Ca). *p14* and *p16* mRNAs showed reduced expression in 84-92% of the bladder tumors; reduced expression of *p15* was documented in 79% of the samples (Figure 12 Ab, Bb & Cb). Surprisingly, no significant association of the mRNA level expression of the studied genes was found with their molecular alterations (Figure 12 Ad, Bd & Cd). Additionally, the studied genes also showed comparable expression levels between AsH vs AsL tumors (Figure 12 Ac, Bc & Cc).

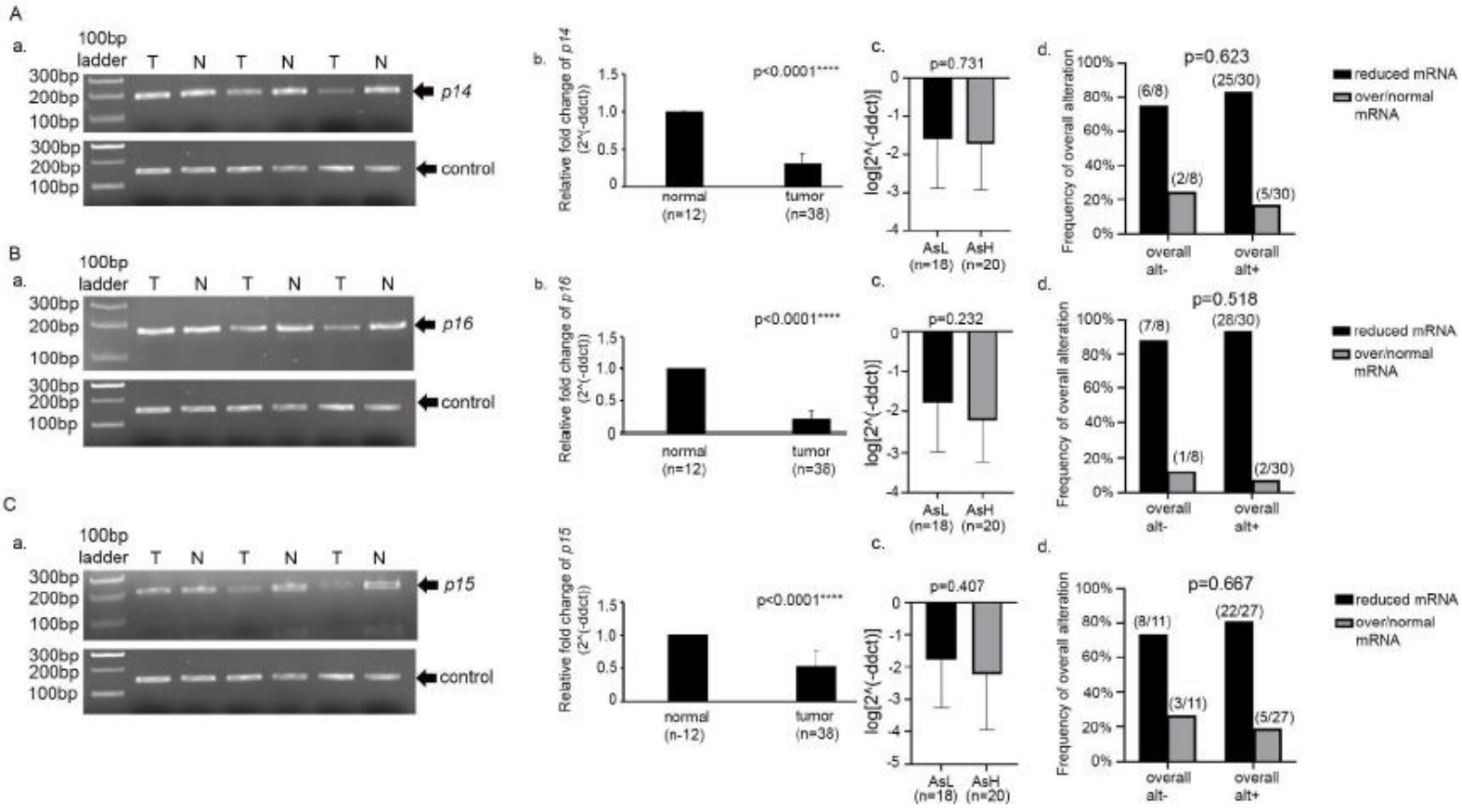


Figure 12: Gene expression at mRNA level. (Aa, Ba, Ca) Representative gel image of mRNA expression of *p14* (200bp), *p16* (180bp), and *p15* (221bp) with internal control, *B2M* (143bp). (Ac, Bc, Cc) Bar diagram showing relative fold change of expression of p14, p16, and p15 in tumor compared to normal tissue. (Ac, Bc, Cc) Association of tissue arsenic with mRNA expression of the three genes. (Ad, Bd, Cd) Association of mRNA expression of the genes with their overall molecular alteration.

(2) Negative regulation of ERBB signaling pathway

Genes involved: *SH3GL2, EGFR*

SH3GL2 involves in receptor-mediated endocytosis of epidermal growth factor receptor (*EGFR*, a member of ERBB family) in association with CIN85; this is following poly-ubiquitination of the receptor molecule (by Cbl, an ubiquitin ligase) and its degradation leading to the cessation of receptor-induced signal transduction process.[65] EGFR overexpression was reported frequently in several malignancies although, protein level expression did not show any correlation with the gene copy number in majority of studies. In HNSCC, overexpression of EGFR was proposed to be mediated via transcriptional inactivation of SH3GL2 by deletion and promoter methylation.[66] In bladder tumor the SH3GL2- EGFR axis has been rarely explored; although expression analysis of SH3GL2 and EGFR in bladder tumor was done so far in one study[67], the molecular alteration of SH3GL2 has not been analysed and no studies has been conducted till date in connection with arsenic-induced bladder cancer.

To understand the role of arsenic-induced alterations (genetic and epigenetic) of SH3GL2 in the expression of EGFR (both mRNA and protein level), following analyses were performed.

Molecular alteration of *SH3GL2*

Deletion analysis:

Deletion of SH3GL2 locus was found in 50% (5/10) of bladder tumor samples included in high resolution CGH-SNP micro-array analysis; deletion frequency in AsH tumors (80%, 4/5) was evidently higher than that of AsL type (20%, 1/5) (Figure 13A). To validate our array-based data, CNV data of SH3GL2 from TCGA-BLCA sample set, and that obtained from deletion analysis in an independent bladder tumor set (n=134) using locus specific (SH3GL2) exonic markers were used. SH3GL2 locus remained deleted in 52% (215/411) of bladder tumors of TCGA dataset (Figure 13B) which was comparable to overall deletion frequency of the locus in our array-based data. In CNV analysis by exonic marker, SH3GL2 was seen to deleted in 61% (82/134) of bladder tumors and concordant to the array-based data, deletion frequency in AsH tumors remained significantly higher than AsL subtype (69%, 52/75 vs. 51%, 30/59; p=0.03) (Figure 13C). Moreover, in our sample set, MIBC samples showed higher frequency of deletion (72%; 34/47) for SH3GL2 than NMIBC (55%; 48/87), but the difference was not statistically significant (p=0.063). (Figure 13C).

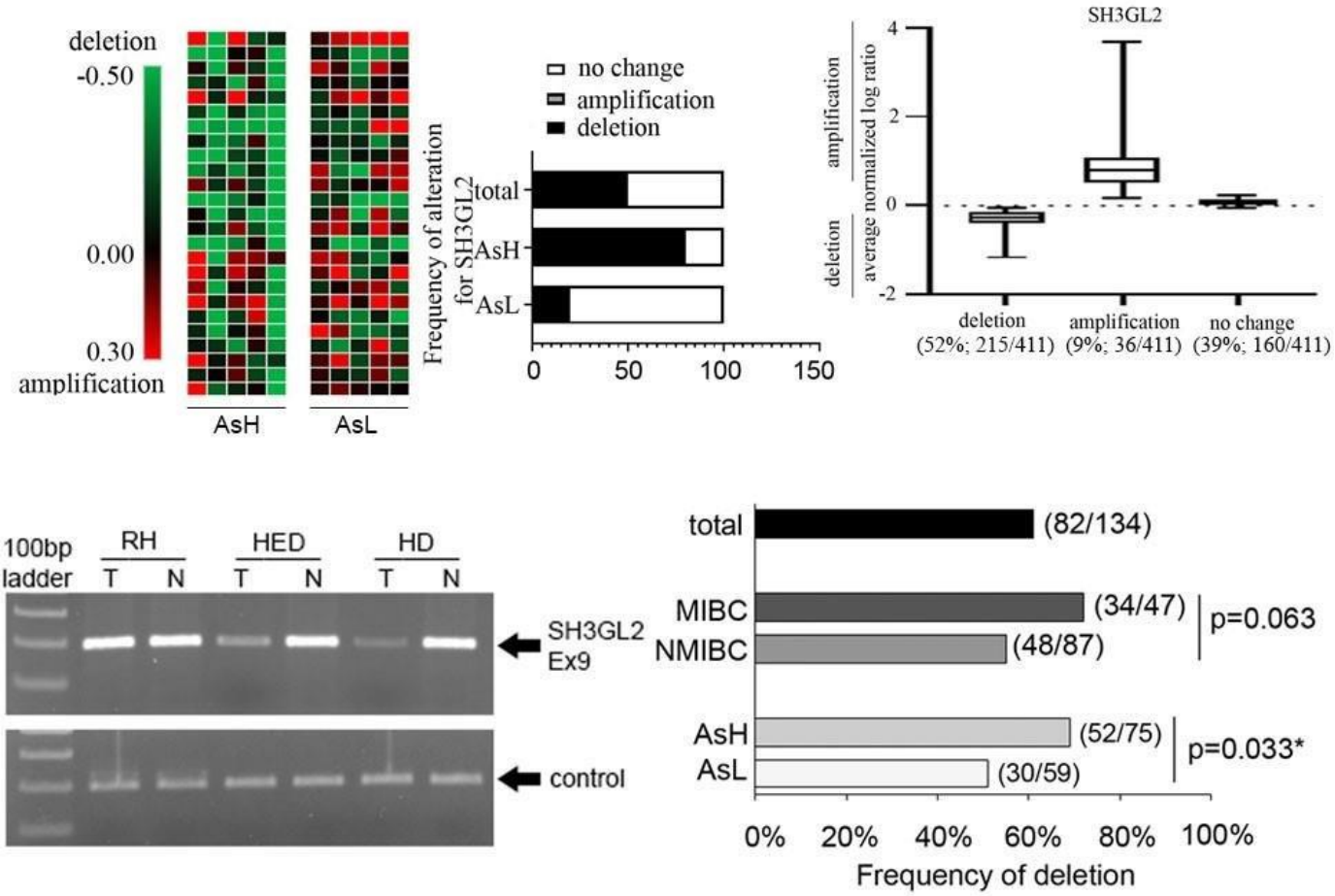


Figure13: Deletion status of *SH3GL2* gene. (A) Deletion status of *SH3GL2* locus in CGH-SNP array data represented in heat-map (a) and summarized view of deletion in array by histogram (b). (B) Alteration of *SH3GL2* locus in TCGA-BLCA dataset. (C) Representative gel photograph showing types of alteration of the genes from exonic-marker based CNV analysis (a) and histogram of frequency of deletion of *SH3GL2* in AsH-AsL and NMIBC-MIBC groups (b).

Promoter methylation analysis:

Apart from deletion, *SH3GL2* also showed promoter methylation in 31% (41/134) of the studied samples. Although, methylation frequency of the gene locus showed an increasing trend in AsH samples compared to AsL (33% vs 27%; p=0.457) and in MIBC samples compared to NMIBC (40% vs 25%; p=0.079), the difference did not reach to statistical significance (Figure 14A).

When gene deletion and promoter methylation were considered together, 70% (94/134) of samples showed overall alteration (deletion/ promoter methylation in at least one of the alleles) for SH3GL2 (Figure 14B). Significantly higher frequency of overall alteration was found in AsH samples compared to AsL (81%, 61/75 vs. 56%, 33/59; p=0.002), while

alteration frequencies remained comparable in NMIBC and MIBC tumors (66%, 57/87 vs 79%, 37/47, p=0.119) (Figure 14B).

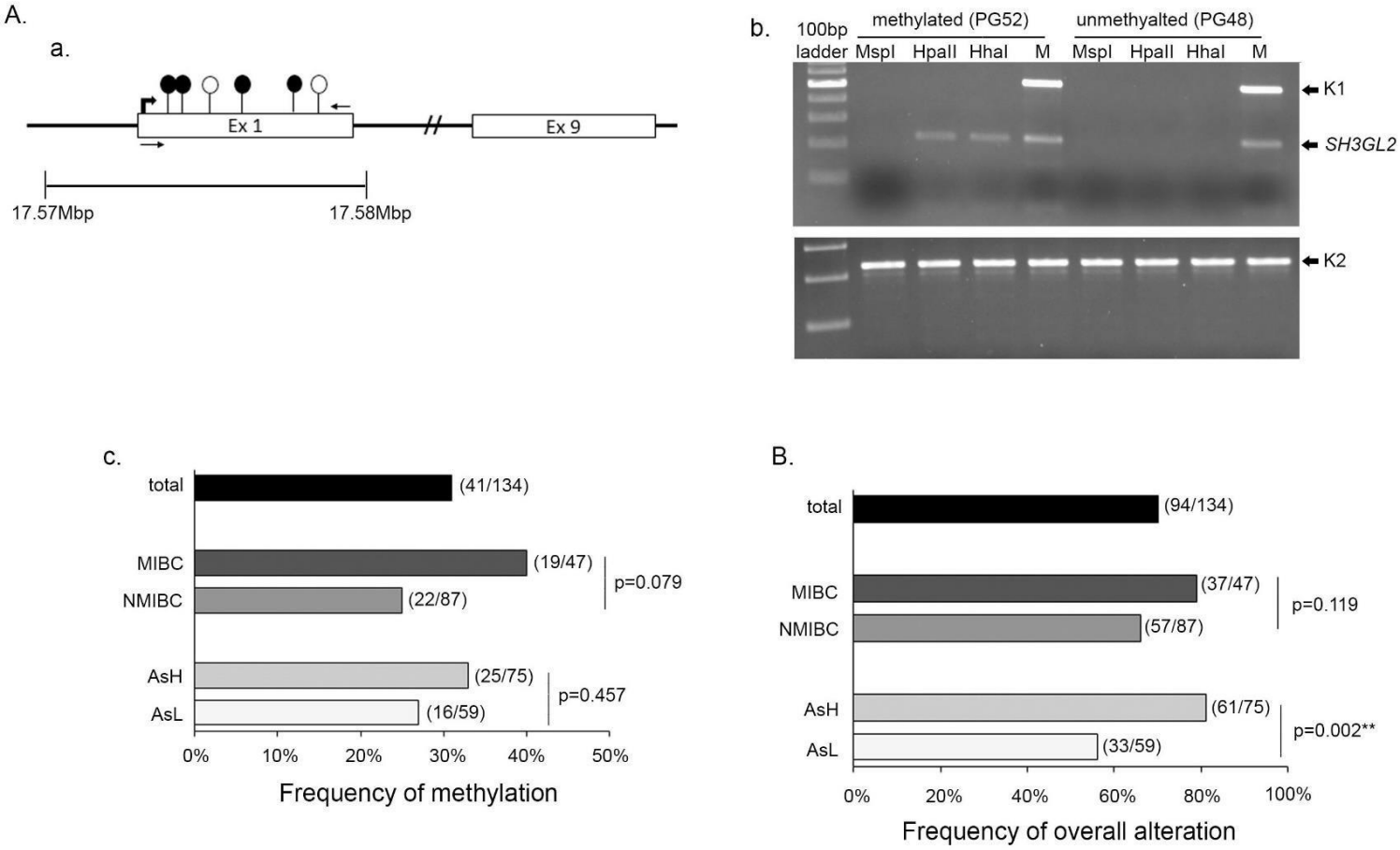


Figure 14: Promoter methylation status of *SH3GL2* gene. (A) Promoter region of *PHF2* showing forward (→) and reverse primer (←), cut-site of the restriction enzymes, MspI & HpaII ♀ and HhaI ● used in MSRA, transcription start site (TSS). Representative gel photograph of MSRA of *SH3GL2* (217bp) where K1 (506bp) and K2 (227bp) served as digestion control and DNA integrity control; MspI and HpaII were methylation sensitive and insensitive restriction digestion enzymes, respectively (a) and histogram showing methylation frequency of the gene AsH-AsL and NMIBC-MIBC groups (b). (B) Histogram showing overall alteration of the gene in the same groups.

mRNA Expression analysis

The mRNA expression of *SH3GL2* was analysed in 38 bladder tumor samples. Reduced level of SH3GL2mRNA was detected in 82% (31/38) of tumor samples compared to the adjacent normal; AsH tumors showed comparatively higher fold reduction of expression than AsL tumors (p=0.09) however, no significant difference in the expression level was found between NMIBC and MIBC (Figure 15A). *SH3GL2* expression was also checked in GEO dataset (GSE13507). Similar to the finding in our samples, bladder tumors of the GEO dataset also showed significantly reduced expression of SH3GL2 compared to

adjacent normal, and the expression remained comparable in NMIBC vs. MIBC samples (Figure 15B).

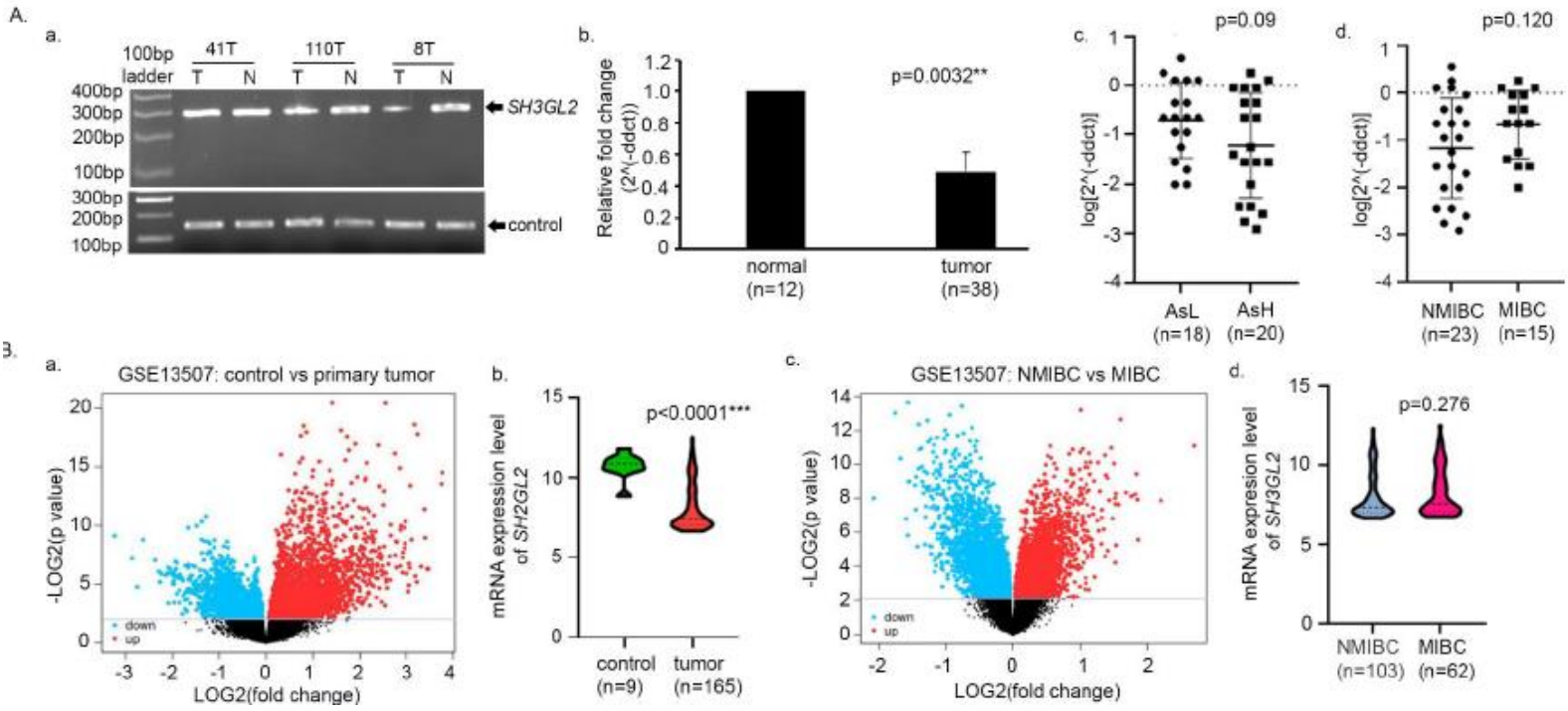


Figure15: mRNA expression of *SH3GL2*. (A) Representative gel photograph of mRNA expression of *SH3GL2* (272bp) along with *B2M* (143bp) (a); bar diagram showing fold change of expression in tumor compared to adjacent normal tissue (b); association of mRNA expression of *SH3GL2* with tumor tissue arsenic (c) and tumor stages (d) (B). mRNA expression level of *SH3GL2* in BLCA from GEO database between tumor vs normal (a&b) and NMIBC vs MIBC (c&d) samples.

Immunohistochemical analysis

SH3GL2 expression was analysed at protein level by immunohistochemistry in 77 bladder tumor (57%, 77/134) depending upon tissue availability. SH3GL2 protein was found to be expressed in the cytoplasm and cell membrane of tumor tissue (Figure 16A). Low/ intermediate level of SH3GL2 protein was detected in 63% (49/77) of bladder tumors. Significantly higher frequency of AsH tumors showed reduced level (low/intermediate) of SH3GL2 protein than AsL (73%, 34/44 vs. 48%, 15/33; p=0.007) and, concordance of the protein level was found with mRNA level expression (p=0.04) and molecular alterations (p=0.03) of the gene locus (Figure 16B).

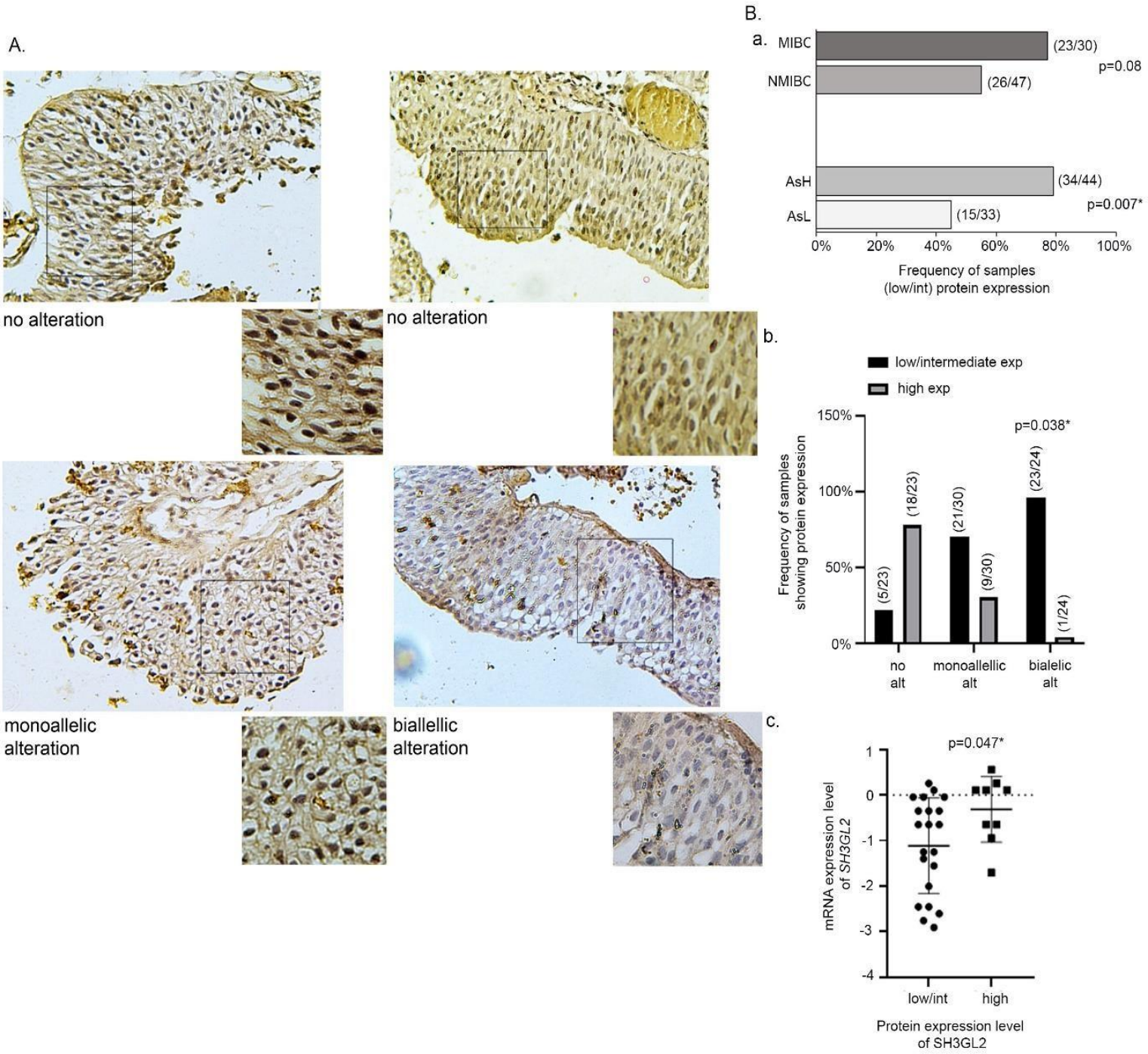


Figure16: Protein expression of *SH3GL2*. (A) Representative IHC of *SH3GL2* in tumor samples. (B) Association of protein expression of *SH3GL2* in AsH and AsL tumors as well as in NMIBC and MIBC samples (a); histogram showing frequency of *SH3GL2* expressing samples along with its molecular alterations (b); association of mRNA and protein expression of *SH3GL2* (c).

Molecular alterations of *EGFR*

To see the functional impact of SH3GL2 down-regulation in bladder tumor, molecular alterations (expression and CNV analyses) of *EGFR*, a downstream target of SH3GL2, was studied on same sample set of our patient population.

mRNA expression analysis

Interestingly, both in our patient samples and GEO dataset (GSE13507), *EGFR* showed a comparable level of expression between primary bladder tumor and adjacent normal tissue (p=0.614 and 0.199 respectively) (Figure 17). In GSE13507, although significantly higher level of EGFR mRNA was found in MIBC samples than NMIBC (p=0.01), no significant difference in the expression level was seen in NMIBC vs. MIBC (p=0.86) and AsH vs. AsL tumors (p=0.85) of our sample set.

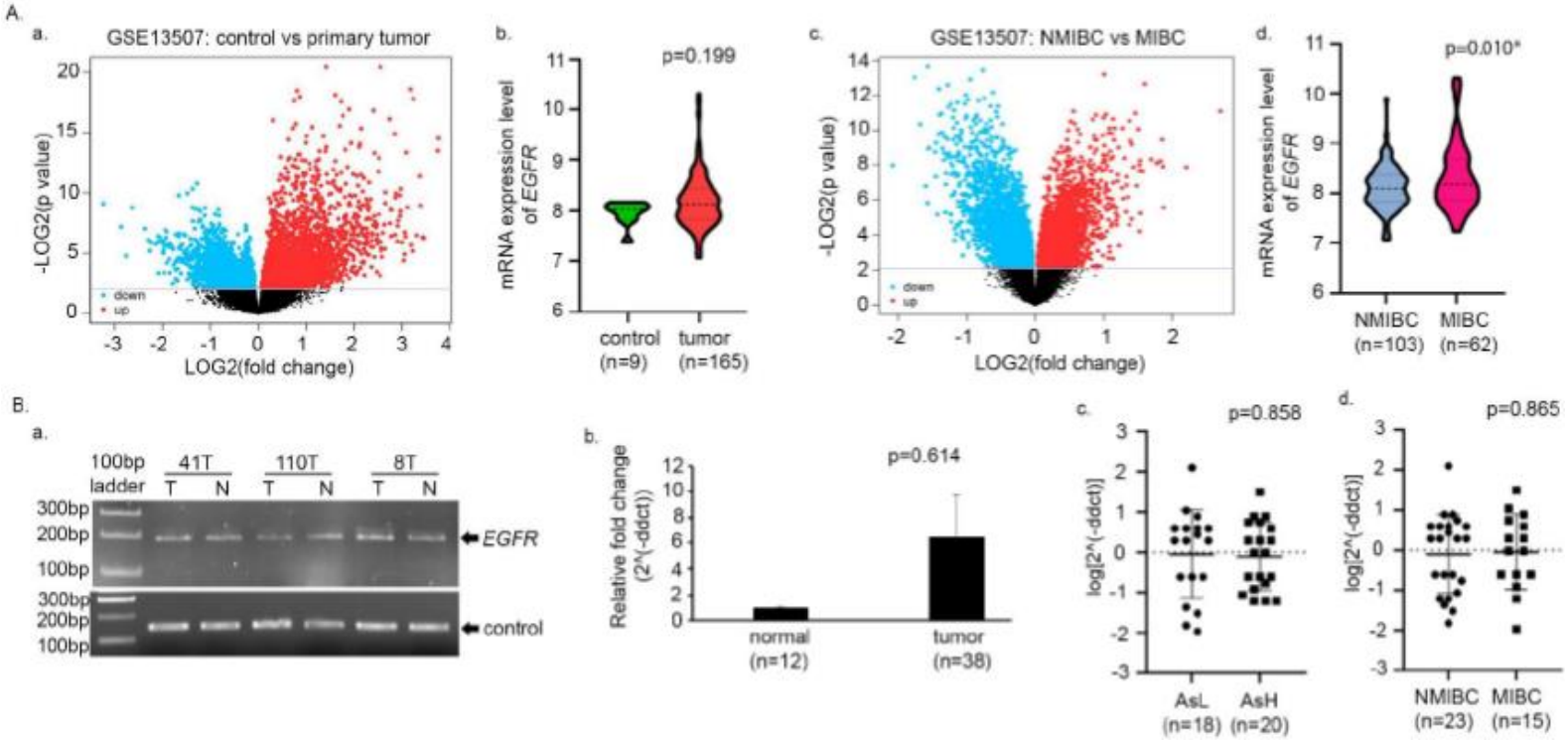


Figure17: mRNA expression of *EGFR*. (A) mRNA expression level of *EGFR* in BLCA from GEO database between tumor vs normal (a&b) and NMIBC vs MIBC (c&d) samples. (B) Representative gel photograph of mRNA expression of *EGFR* (189bp) along with *B2M* (143bp) (a); bar diagram showing relative fold change of expression in tumor compared to adjacent normal tissue (b); association of mRNA expression of *EGFR* with tumor tissue arsenic (c) and tumor stages (d).

Immunohistochemical analysis

Both cytosolic and membranous expression of EGFR protein was detected in bladder tumor samples (Figure 18A). EGFR over-expression was seen in 57% (44/77) of tumor samples analysed and surprisingly, no concordance (p=0.965) was found between mRNA and protein levels expression of the gene in common sample set (n=30) (Figure 18B).

However, similar to mRNA expression, EGFR protein also showed comparable level of expression between NMIBC and MIBC (p-0.09), and, comparatively higher level of expression of the protein in AsH than AsL tumors (p=0.01).

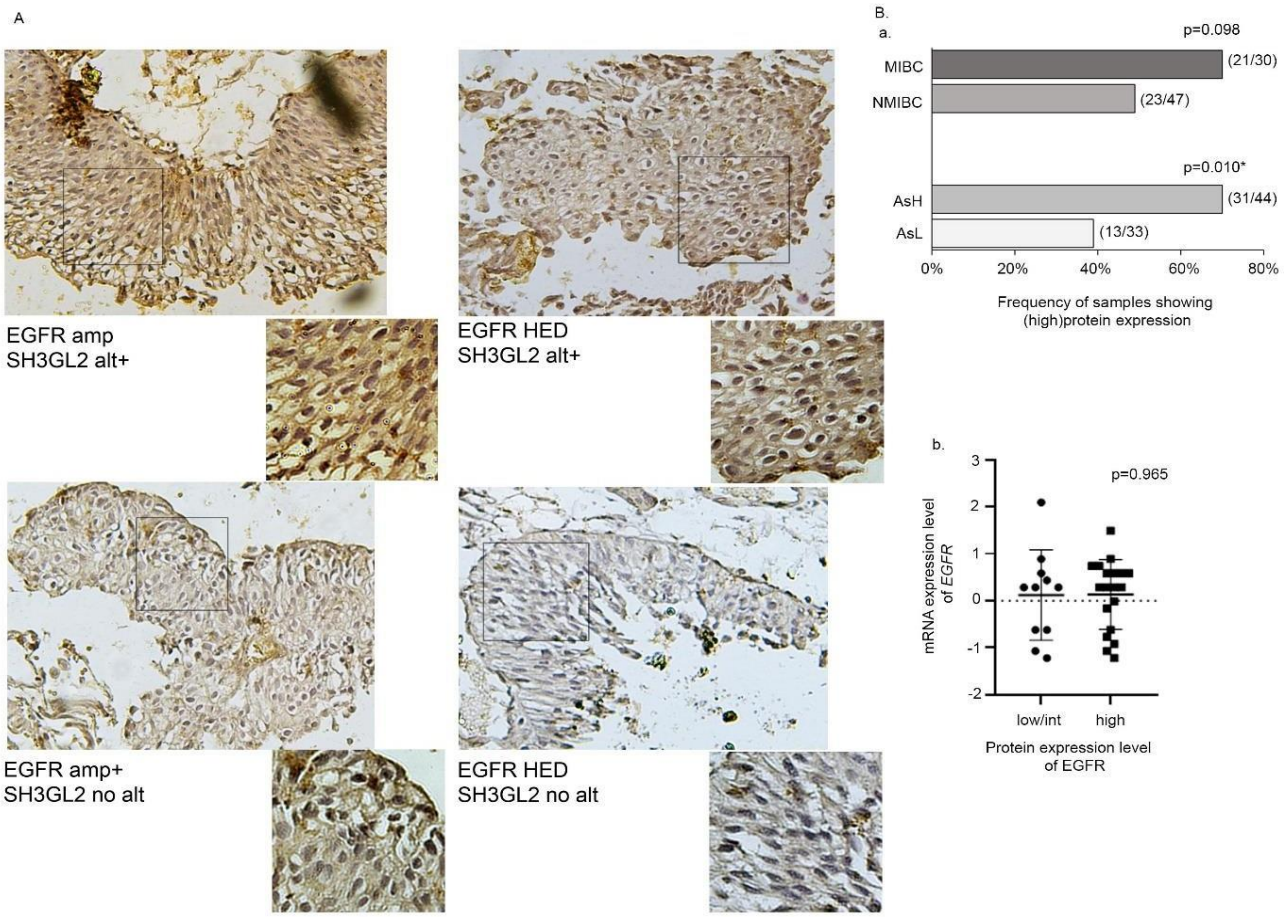


Figure18: Protein expression of *EGFR*. (A) Representative IHC of *EGFR* in tumor samples. (B) Association of protein expression of *EGFR* in AsH and AsL tumors as well as in NMIBC and MIBC samples (a); histogram showing frequency of *EGFR* expressing samples along with its molecular alterations (b); association of mRNA and protein expression of *EGFR*.

CNV analysis

In our CGH+SNP array, infrequent incidence of amplification and deletion of *EGFR* locus (10%, 1/10 for each) was detected. Similarly, in bladder tumors of TCGA dataset, only 8% (34/411) deletion and 23.4% (96/411) amplification was found (Figure 19A&B). In exonic marker-based CNV analysis for validation of array CGH data, we found only 3% (4/134) amplification, but 19% (26/134) deletion, all of hemizygous category, of EGFR (Figure 19C). However, no significant association of EGFR protein was found with the CNV status of the gene locus (p=0.074). Surprisingly, a highly significant negative correlation was found between EGFR and SH3GL2 at the protein level (r=-0.733, p<0.0001) but, such association of EGFR did not exist at the mRNA level with SH3GL2 protein (r=0.102, p=0.591) in our samples (Figure 19D).

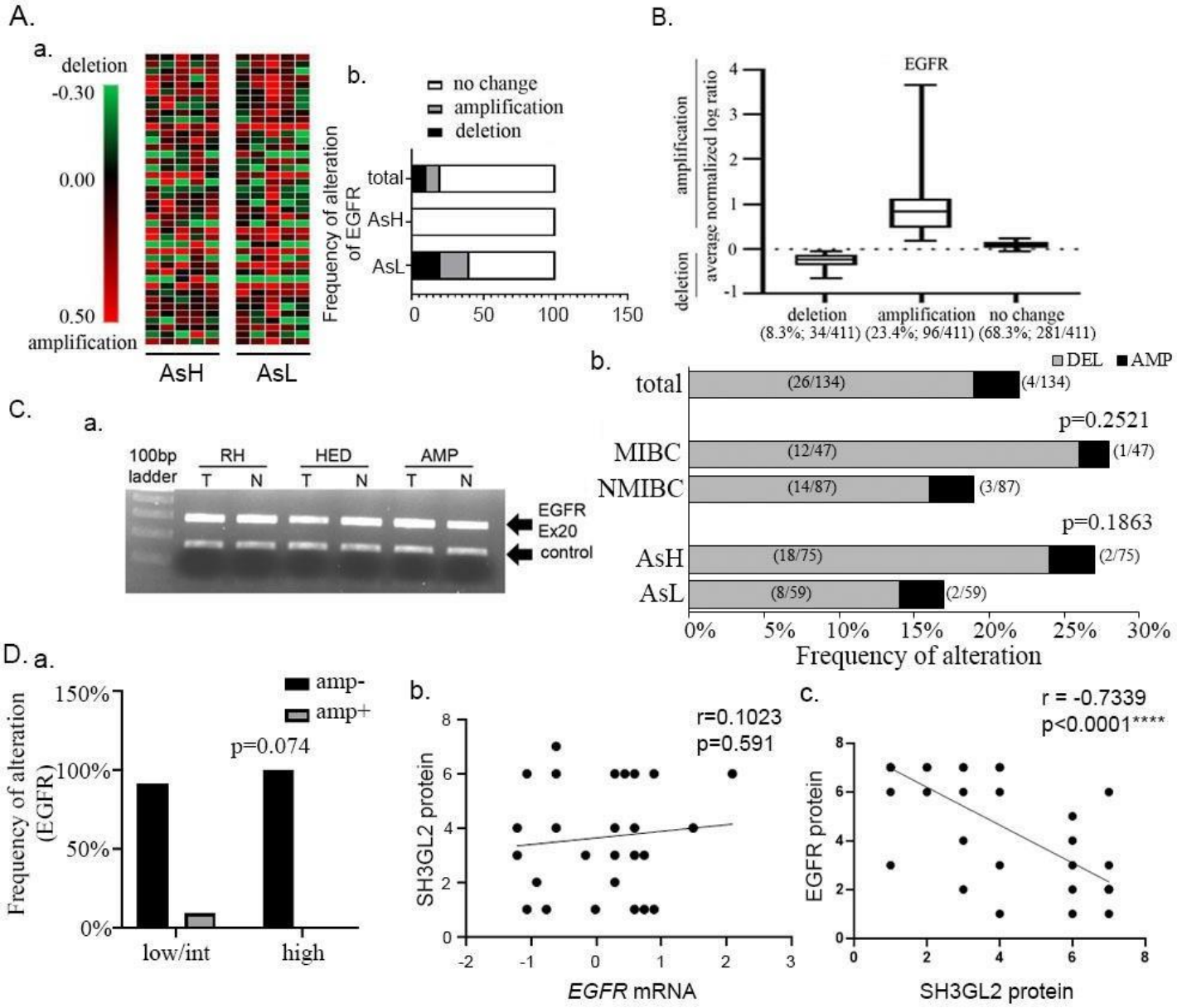


Figure 19: Deletion status of *EGFR* gene. (A) Deletion status of *EGFR* in CGH-SNP array data represented in heat-map (a) and summarized view of deletion in array samples in histogram (b). (B) CNV data of *EGFR* in TCGA-BLCA sample set. (C) Representative gel photograph showing types of alteration of the genes from CNV analysis (a) and histogram of frequency of deletion of *SH3GL2* in AsH-AsL and NMIBC-MIBC groups (b). (D) Association of EGFR protein with its alteration (CNV) (a), SH3GL2 protein with *EGFR* mRNA (b), and between proteins of SH3GL2 and EGFR (c).

Clinicopathological association and patient outcome

In Kaplan-Meier analysis, promoter methylation of *SH3GL2* showed significant association with poor patient survival (p=0.003); the overall alteration (deletion/methylation) status of the gene locus also showed the similar trend (p =0.02) (Figure 20). When samples were categorized based on expression levels (protein) of SH3GL2 and EGFR, patients having "low/intermediate SH3GL2 and high EGFR in tumor showed significant poor outcome than high SH3GL2, low/intermediate EGFR group (p=0.001); due to inadequate sample size, patients showing low levels for SH3GL2 and EGFR (n=6), and high level for both proteins (n=1) were excluded from the analysis (Figure 20).

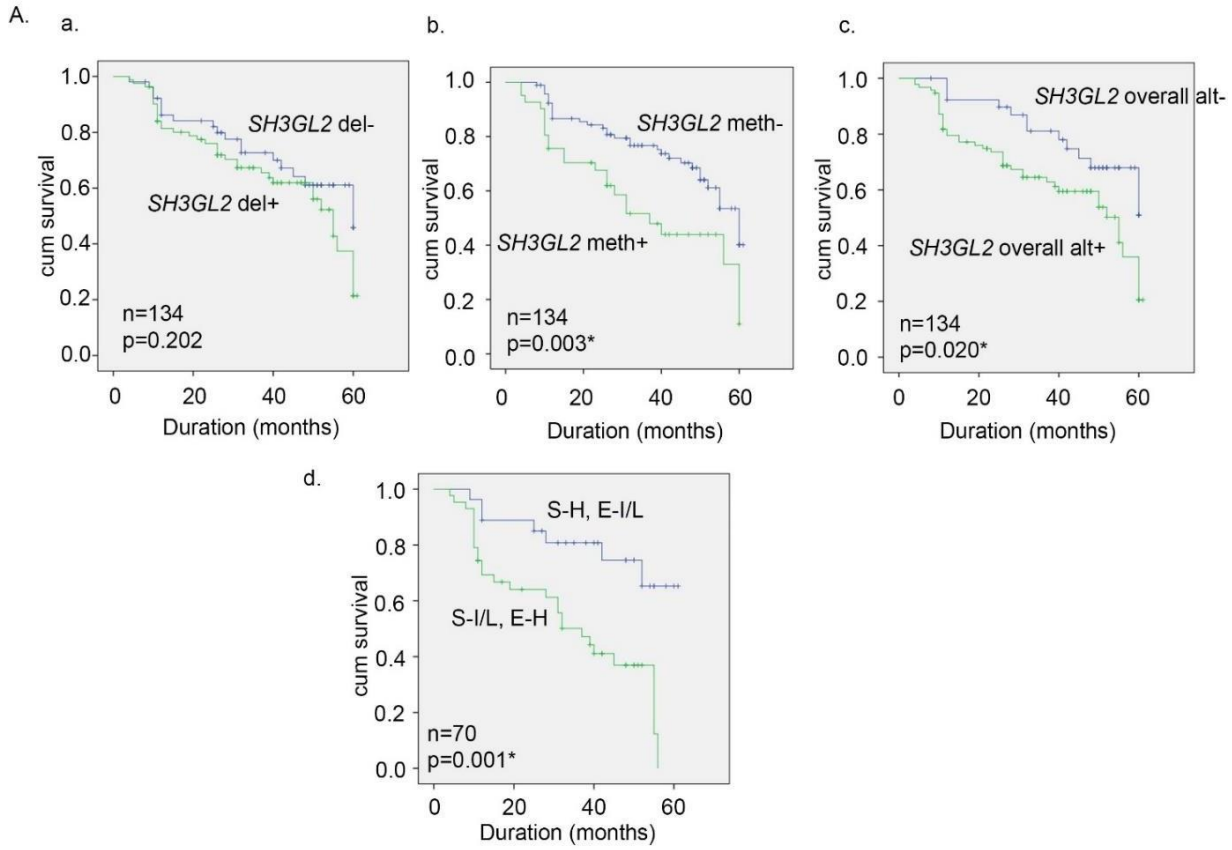


Figure 20: Survival analysis of studied patients. (A) Kaplan-Meier survival analysis curves in association with deletion, methylation and overall alteration of *SH3GL2*, and co- expression of SH3GL2 & EGFR protein. S-H, E-I/L; SH3GL2 high, EGFR intermediate/low, S-I/L, E-H; SH3GL2 intermediate/low, EGFR high.

Histone lysine demethylation and associated transcriptional program

Genes involved: *PHF2, p53, p21*

PHF2, an H3K9-Me2 demethylase was predicted to serve as a candidate TSG in breast and head and neck cancer.[68-69] In colon and stomach cancer, functional characterization of PHF2 was done;[70] PHF2 was found to remove an inhibitory methylation mark from lysine (K9) residue of H3 histone at the p21 promoter following its active transcription mediated by p53. Thus, PHF2 was proposed to exert its tumor suppressive impact through serving as a co-activator of p53 mediated transcriptional expression of downstream target genes. The role of *PHF2* in the pathogenesis of bladder cancer has not been explored to date.

For the first time, we analysed molecular alterations (deletion, methylation and expression) of *PHF2* in arsenic induced bladder cancer, associated the alterations with disease progression and prognosis, and also assess the functional impact of such molecular event on p53 mediated gene (p21) transcription.

Deletion analysis by microsatellite/ exonic markers:

In CGH+SNP microarray, PHF2 deletion was found in 40% (4/10) of the bladder tumor samples. When tumors were categorized based on tissue arsenic level, deletion frequency was shown to be considerably higher in AsH samples (60%, 3/5) compared to AsL ones (20%, 1/5), indicating a potential association of the alteration with arsenic exposure (Figure 21A). The array data obtained from small sample size was further validated by microsatellite/ exonic marker-based deletion mapping in independent set of bladder tumor samples. Deletion of *PHF2* locus was documented in 38% (51/134) of bladder tumors. Consistent with the array-based data, significantly higher deletion frequency was seen in AsH samples compared to AsL (49%, 37/75 vs. 24%, 14/59; p=0.0039). However, NMIBC and MIBC samples showed comparable deletion frequency for the gene locus (40%, 35/87 vs. 34%, 16/47 p=0.576) (Figure 21C). Additionally, the copy number variation (CNV) data of bladder cancer was also downloaded from TCGA database and, the analysis showed comparable high frequency of deletion (42%, 173/411) for *PHF2* similar to the finding in our samples (Figure 21B).

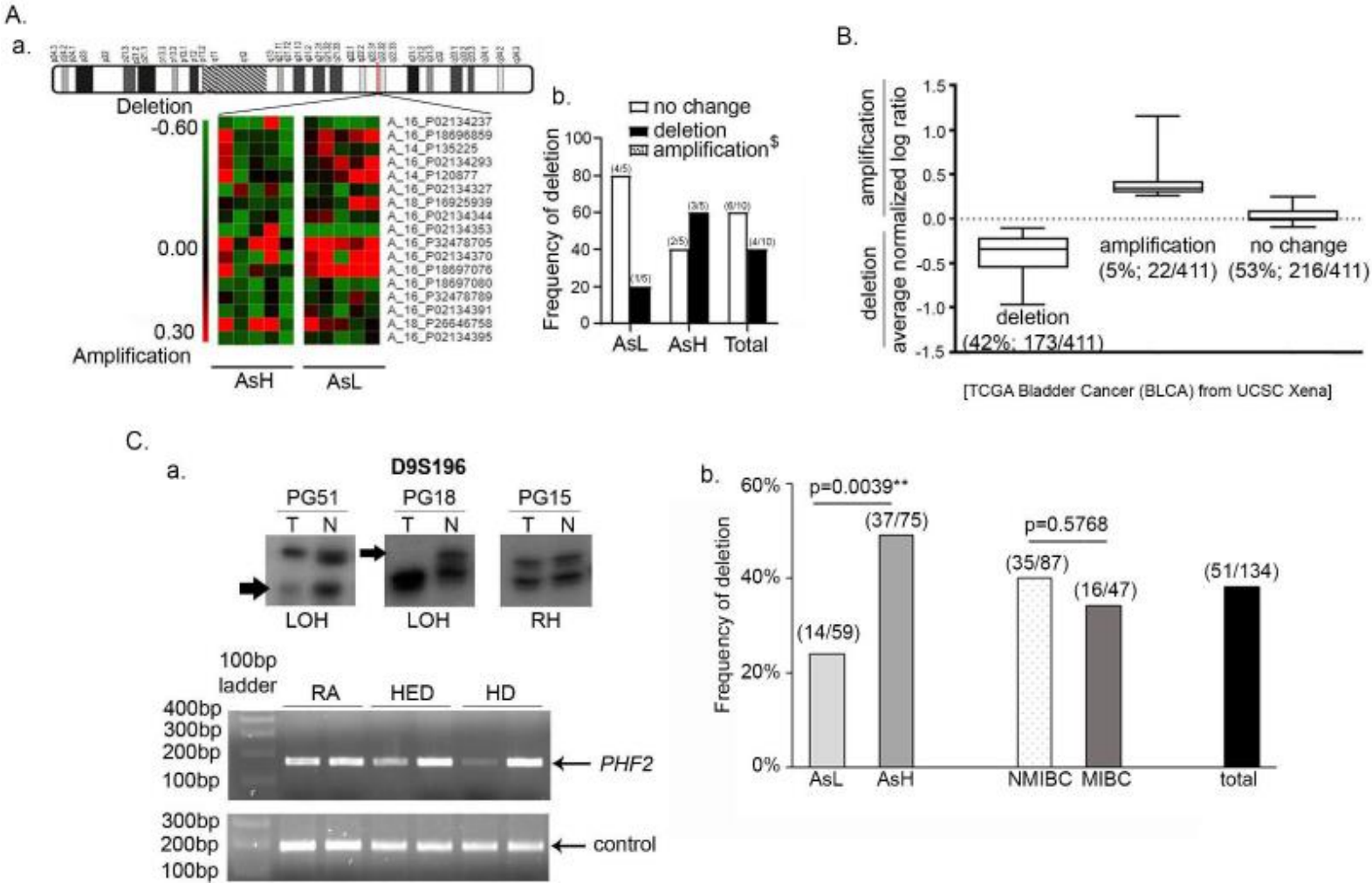


Figure 21: Deletion status of *PHF2* gene. (A) Deletion status of *PHF2* in CGH+SNP array showing in the heat-map (a) and summarized in histogram (b). (B) Box-plot showing the CNV data of the gene according to TCGA database of BLCA. (C) Alterations of *PHF2* recorded from microsatellite and exonic marker-based CNV analysis in our sample (a), frequency of deletion of samples in AsH-AsL and NMIBC-MIBC groups (b).

Promoter methylation analysis by MSRA:

In MSRA analysis, 51% (69/134) of the bladder tumor samples showed methylated promoter for *PHF2* locus. We have also analysed *PHF2* methylation in adjacent normal samples of 42 tumors; interestingly, in this common sample set, 22 (52%) tumors showed methylated *PHF2* promoter, of which in 14 (64%), gene methylation was detected in the adjacent normal tissue also (Figure 22). Consistent to the deletion analysis, significantly higher frequency of *PHF2* promoter methylation was seen in AsH tumors than AsL ones

(67%, 50/75 vs. 32%, 19/59; p=0.0001) and, comparable frequency among NMIBC and MIBC samples (47%, 41/87 vs. 60%, 28/47; p=0.2059) (Figure 22).

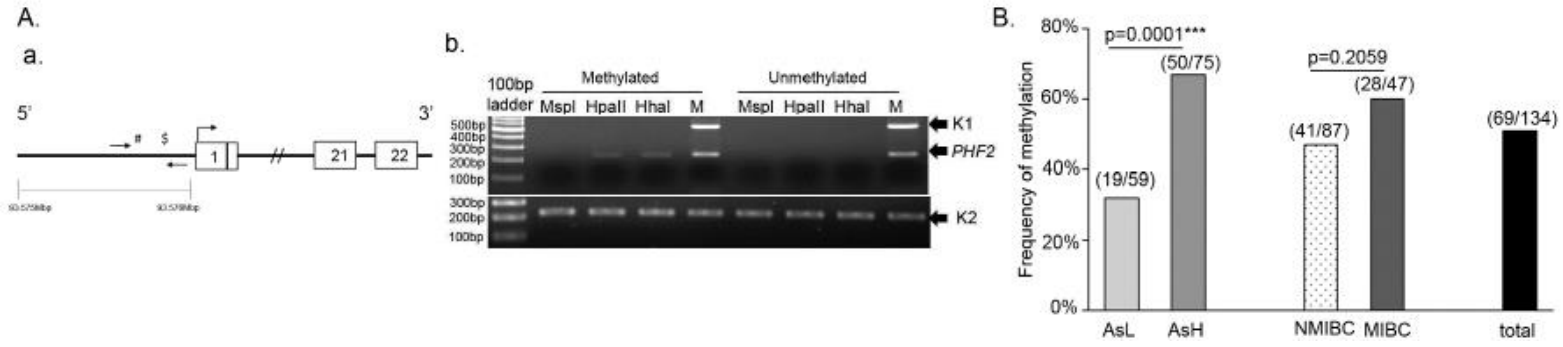


Figure 22: Promoter methylation analysis of *PHF2* gene. (A) Promoter region of *PHF2* showing forward (→) and reverse primer (←), cut-site of the restriction enzymes, MspI & HpaII (#) and HhaI ($) used in MSRA, transcription start site (TSS) (a). Representative gel photograph of MSRA of *PHF2* (223bp) along with K1 (506bp) and K2 (227bp) (b), (B) Frequency of methylation of samples in AsH-AsL and NMIBC-MIBC groups

mRNA expression profile of *PHF2*

mRNA expression analysis of *PHF2* was done in 38 primary bladder tumor samples by qRT-PCR. Reduced PHF2mRNA level was detected in 89% (34/38, p<0.0001) of tumors than adjacent normal bladder tissue. AsH tumors showed significantly low expression of the gene than AsL (p=0.0431) and, in NMIBC and MIBC samples, the expression did not differ considerably (p=0.4453). To validate our finding, mRNA expression data of *PHF2* in bladder tumors of TCGA and GEO (GSE128959) datasets was analysed. Similar to the findings in our samples, the analysis showed significantly reduced PHF2mRNA expression in bladder tumors than in adjacent normal (TCGA dataset, p=0.012) and comparable

expression levels along different tumor stages (stage-I vs. stage-II vs. stage-III vs. stage-IV and NMIBC vs MIBC in TCGA and GEO datasets) (Figure 23).

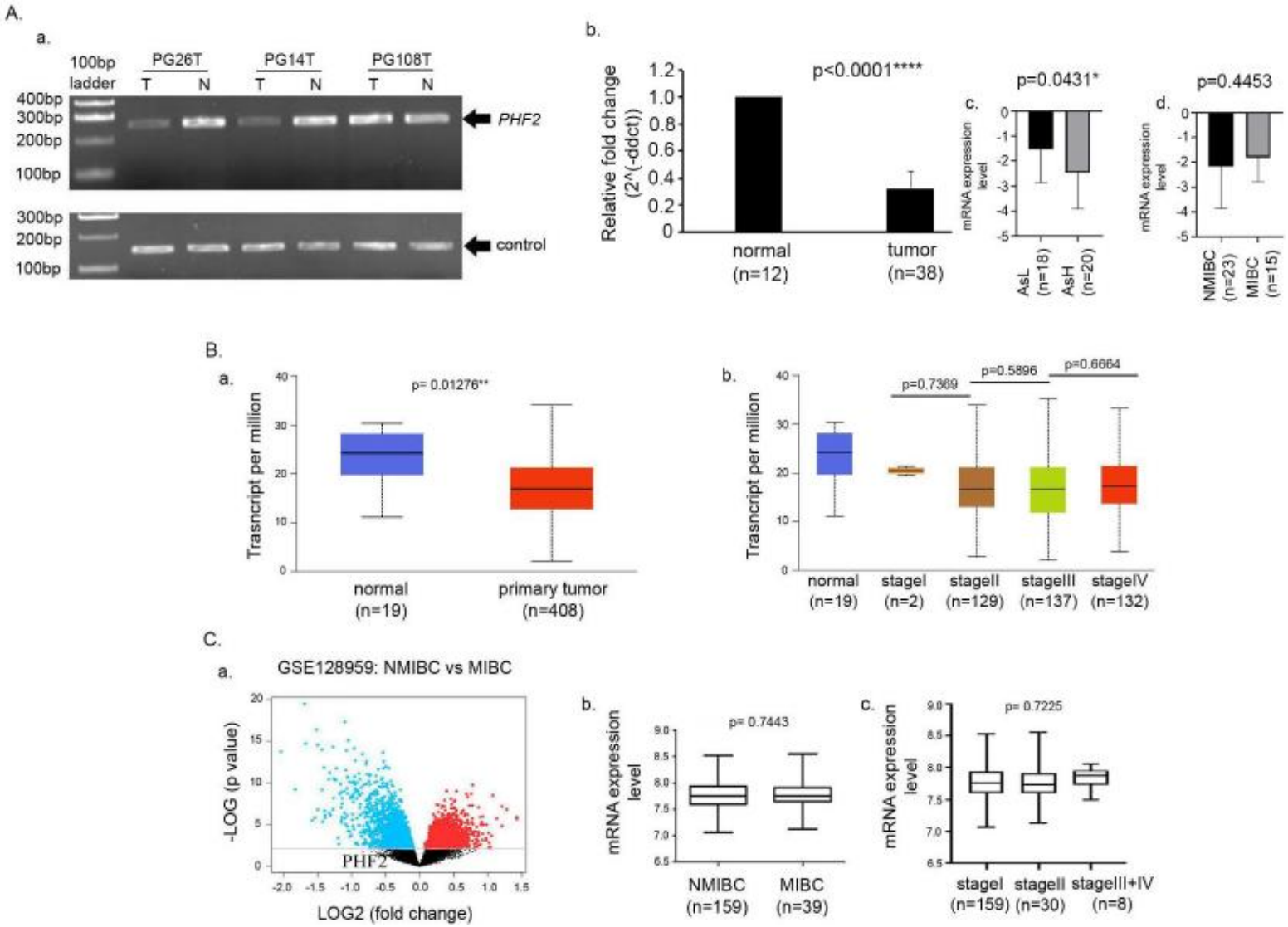


Figure 23: Expression pattern of *PHF2* at mRNA level. (A) Representative gel photograph of mRNA expression of *PHF2* (258bp) along with *B2M* (143bp) (a); association of mRNA expression of *PHF2* with tumor tissue arsenic (b) and tumor stages (c). (B) mRNA expression level of PHF2 in BLCA from TCGA and (C) GEO database.

Immunohistochemical profile of *PHF2*

Distinct nuclear expression of PHF2 protein was detected in normal urothelium (tumor adjacent) and bladder tumors (Figure 24A). 68% (50/73) of tumor samples showed reduced (low/intermediate) PHF2 expression and among these, AsH tumors were significantly more frequent than AsL ones (87.5% vs. 45%, p=0.0001), but, the distribution of NMIBC and MIBC samples remained proportional (Figure 24A&B). Concordance was found (p=0.03) between mRNA and protein level expression of PHF2 among the common sample set

(n=30) in which both analyses were done (Figure 24C). A significant association of reduced PHF2 expression was also found with mono and bi-allelic alterations (deletion/methylation) of the gene locus (p=0.05 & 0.0003, respectively) (Figure 24D).

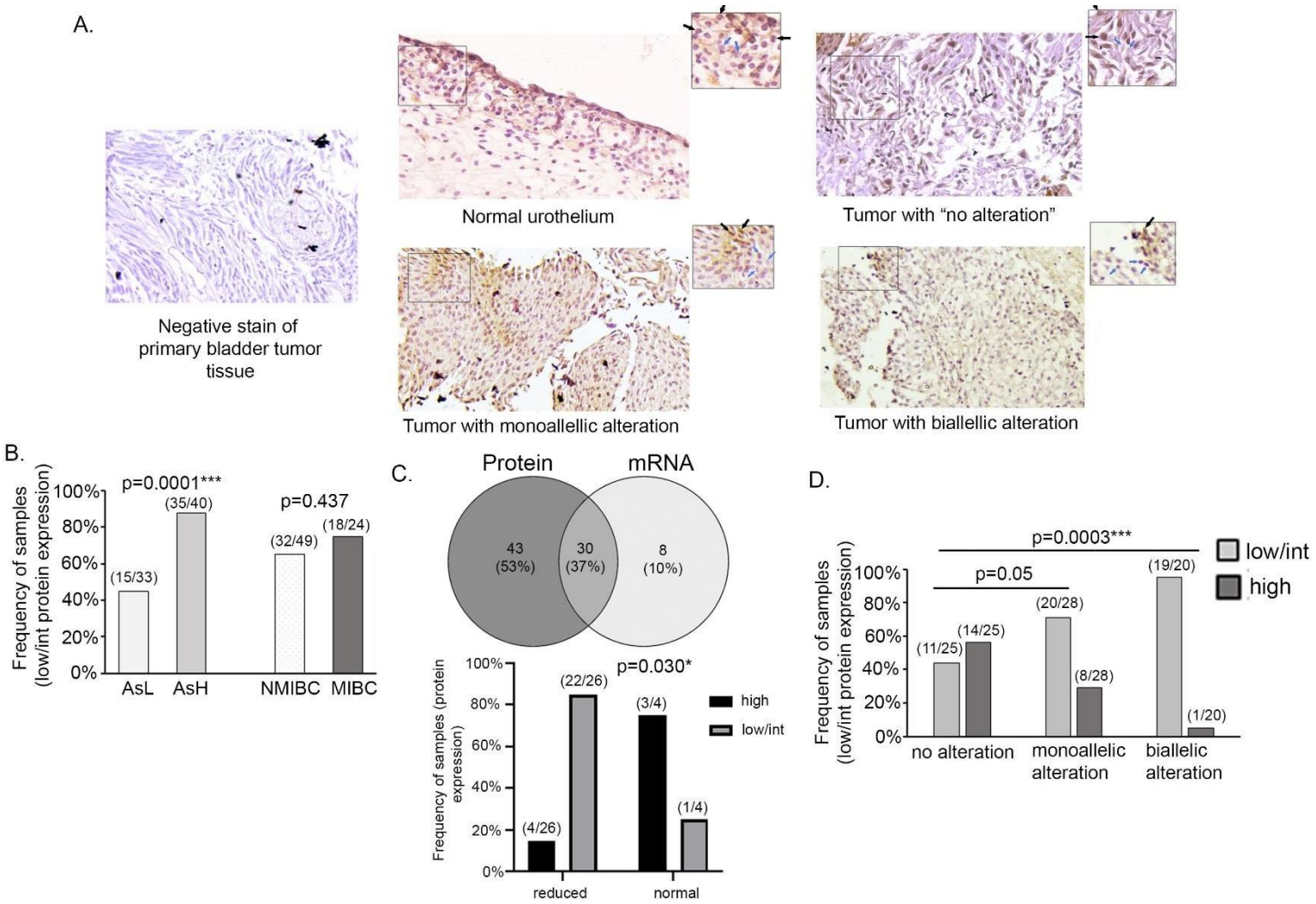


Figure 24: Protein expression of PHF2 in BLCA. (A) Representative photograph of immunohistochemistry of PHF2 in normal urothelium, tumor without genetic alteration, with monoallellic and biallellic alteration (B) Distribution of samples expressing PHF2 protein in AsH-AsL and NMIBC-MIBC groups (C) Distribution of samples expressing PHF2 protein that were analysed for both mRNA and protein level. (D) Association of protein expression with genetic alterations of the samples.

Molecular alterations of p21 and

p53 mRNA expression analysis

mRNA expression analysis of *p21* and *p53* was done on the same set of bladder tumor samples (n=38) in which *PHF2* expression (mRNA) was analysed. Significantly reduced expression of *p21* was detected in tumors compared to adjacent normal tissue (p=0.0007); interestingly, in this sample set, p53mRNA level was found to remain upregulated than the adjacent normal and, the difference was statistically significant (p=0.0004) (Figure 25A&B). Moreover, out of a total 32 bladder tumors showing reduced expression for *p21*, downregulation of PHF2 (mRNA) was detected in 31 (82%, p=0.008) and of p53 in only 8 (25%, p=0.3) samples (Figure 25C). Consistent to our finding, significantly downregulated expression of *p21* and upregulated expression of *p53* was also documented in bladder tumor samples of TCGA dataset (Figure 25D).

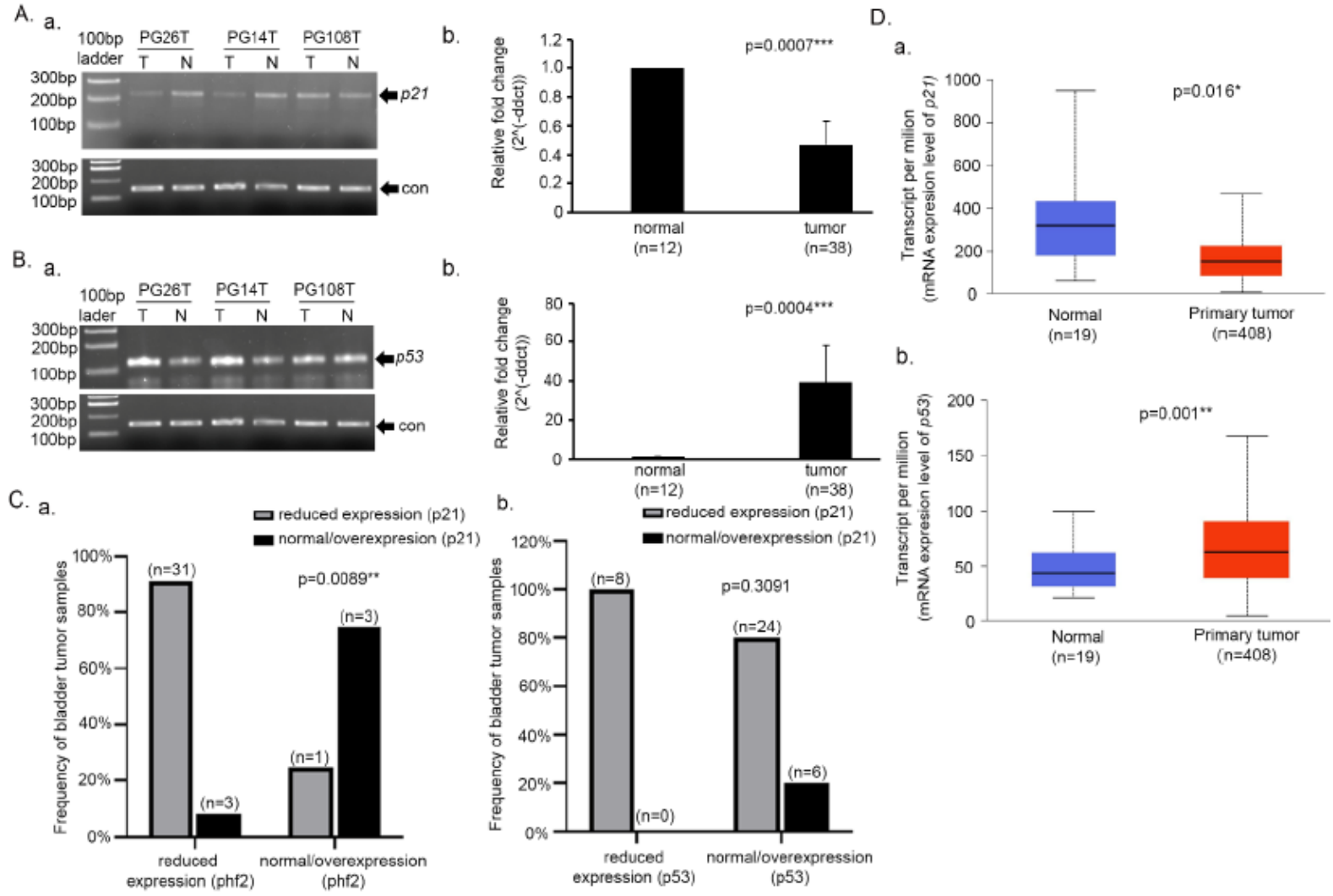


Figure 25: Expression pattern of *p21* and *p53* at mRNA level. (Aa, Ba) Representative gel photograph of mRNA expression of *p21* (215bp) and p53 (125bp) along with *B2M* (143bp). (Ab, Bb) Bar diagram of relative fold change of expression of *p21* and *p53* in tumor compared to adjacent normal tissue. (C) Association of mRNA expression of *p21* with the mRNA level of (a) *PHF2* and (b) *p53*. (D) mRNA expression level of *p21* (a) and *p53* (b) in BLCA from TCGA database Analysis of p21 and p53 proteins

Immunohistochemical analysis of p21 and p53 proteins was done on the same set of bladder tumors (n=73) in which PHF2 protein was analysed. Predominantly nuclear expression for both p21 and p53 proteins was seen in normal urethelium (tumor adjacent) (Figure 26A). Consistent to the mRNA expression profile, reduced expression level (low/ intermediate) of p21 protein was detected in 77% (56/73) of bladder tumor samples; while, for p53, low/intermediate expression was seen in 37% (27/73) of tumors and, in the remaining 63%, high expression level of the protein was scored (Figure 26A&B). In the association study, p21 protein in bladder tumors showed a highly significant positive correlation (r=0.8, p<0.0001) with PHF2; however, the correlation between p21 and p53 proteins was found to be week and the strength was not statistically significant (r=0.12, p=0.29) (Figure-26C).

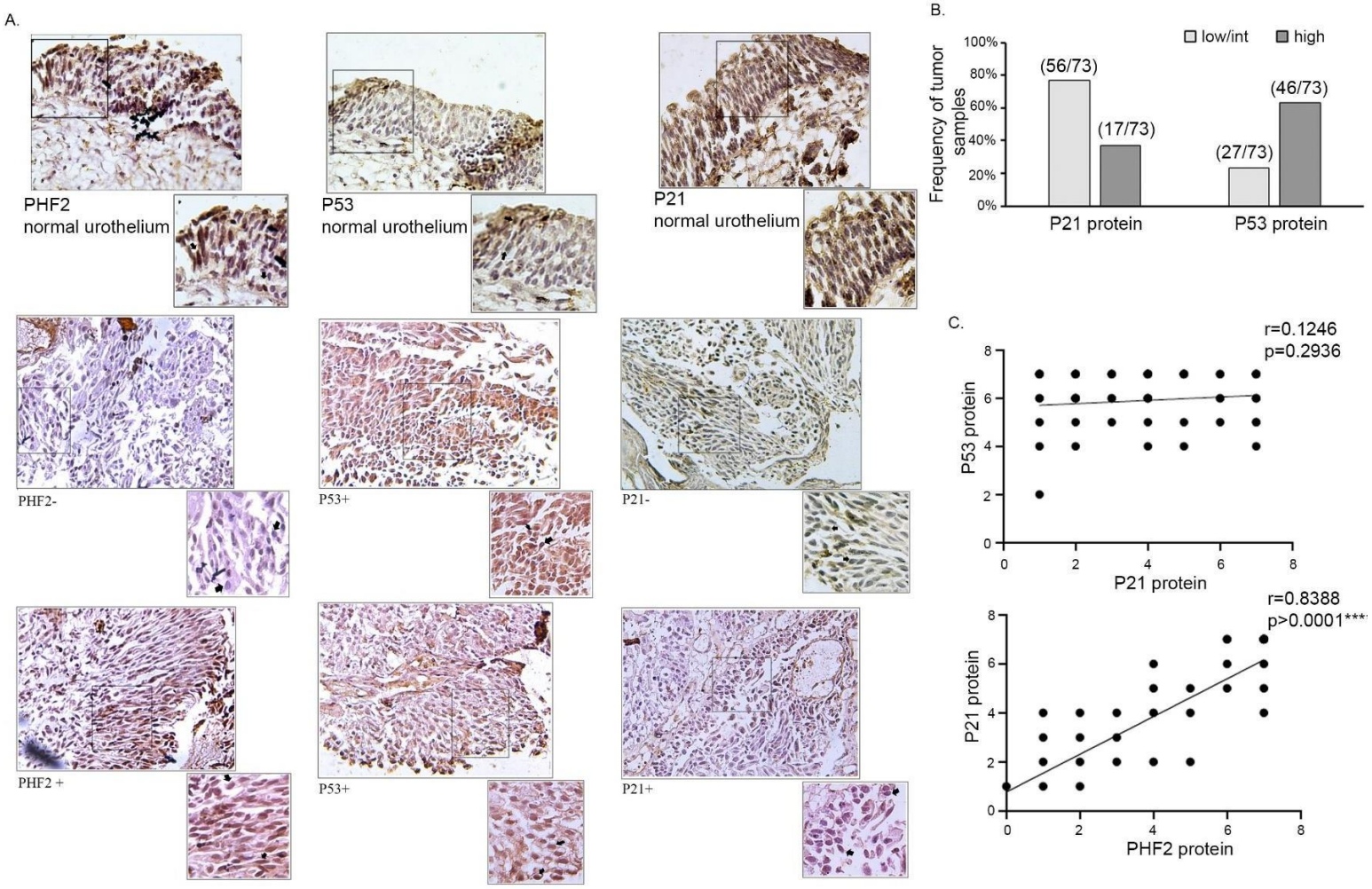


Figure 26: Correlation of expression of the three studied proteins. Comparison of protein expression pattern of PHF2 (A), P53 (B) and P21 (C) in normal urothelium, tumor tissues. (B) Histogram showing frequency of samples expressing P21 and P53 protein. (C) Correlation of expression of proteins: P53 vs P21 and P21 vs PHF2.

Deletion and promoter methylation analyses of *P21*

The analyses were done in a total of 134 bladder tumor samples to assess the impact of genetic and epigenetic alterations at the gene locus (*p21*) on its expression. Deletion and promoter methylation of *p21* was studied by differential PCR and MSRA in our bladder tumor samples (Figure-27A). *P21* gene locus showed comparatively low frequency of deletion (19%, 25/134) and promoter methylation (17%, 23/134) in bladder tumors (Data not shown). When deletion and/ or methylation was considered, the overall alteration frequency of *p21* locus was found to be 29% that did not differ significantly in AsH vs. AsL tumors (p=0.704) and in NMIBC vs. MIBC samples (p=0.23) (Figure 27B). Interestingly in the common sample set, no significant association of the reduced p21 expression at mRNA and protein levels was found with the alteration status of the gene (Figure 27C). significant association of the reduced p21 expression at mRNA and protein levels was found with the alteration status of the gene (Figure 27C).

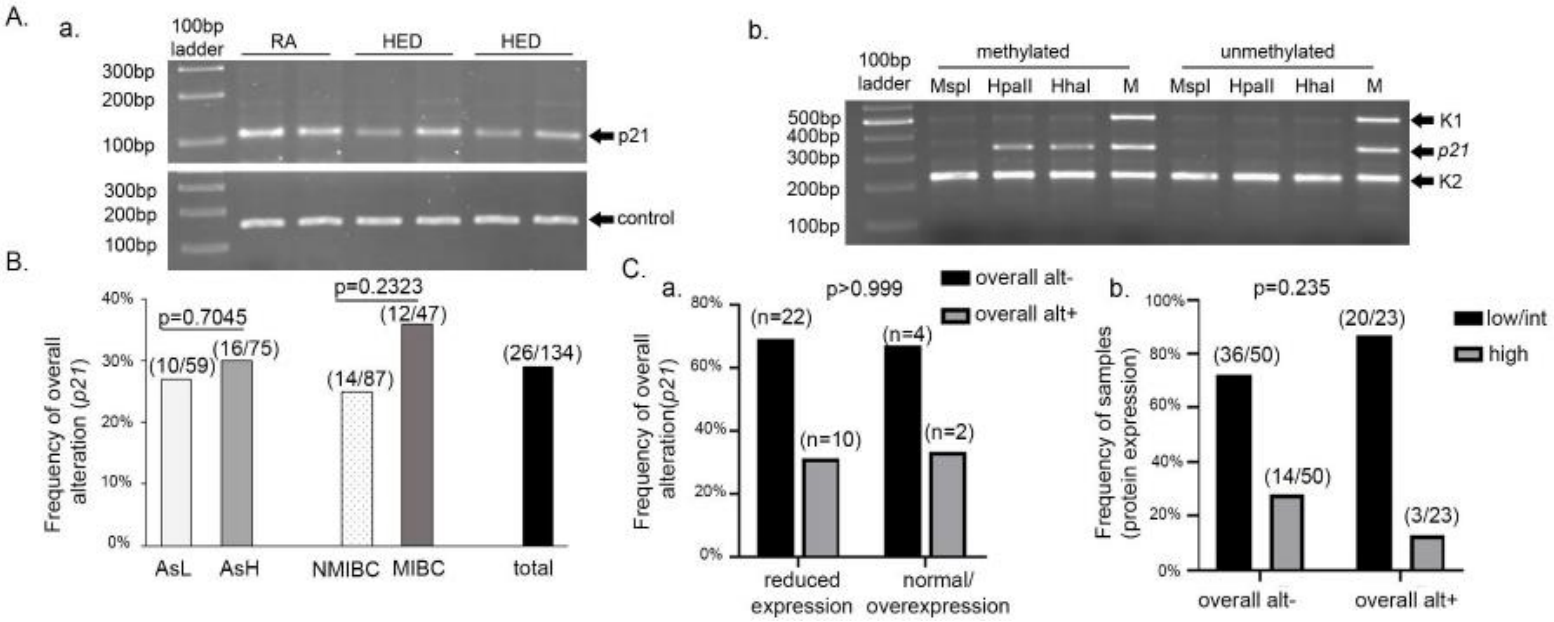


Figure 27: Molecular alteration status of *p21* and its association with mRNA expression. (A) Representative gel photograph of deletion status of p21 (112bp) (a) and methylation status of *p21* (334bp) along with K1 (506bp) and K2 (227bp) (b). (B) Frequency of overall alteration of *p21* in AsH-AsL and NMIBC-MIBC groups. (C) Association of mRNA expression and protein expression level of *p21* with its own overall molecular alterations.

Patient survival

A total of 134 bladder cancer patients were included in the analysis. In Kaplan-Meier analysis, a significant association of deletion (p=0.002), promoter methylation (p=0.01), and overall alteration (p=0.001) of *PHF2* was found with poor overall patient survival (Figure 28A).

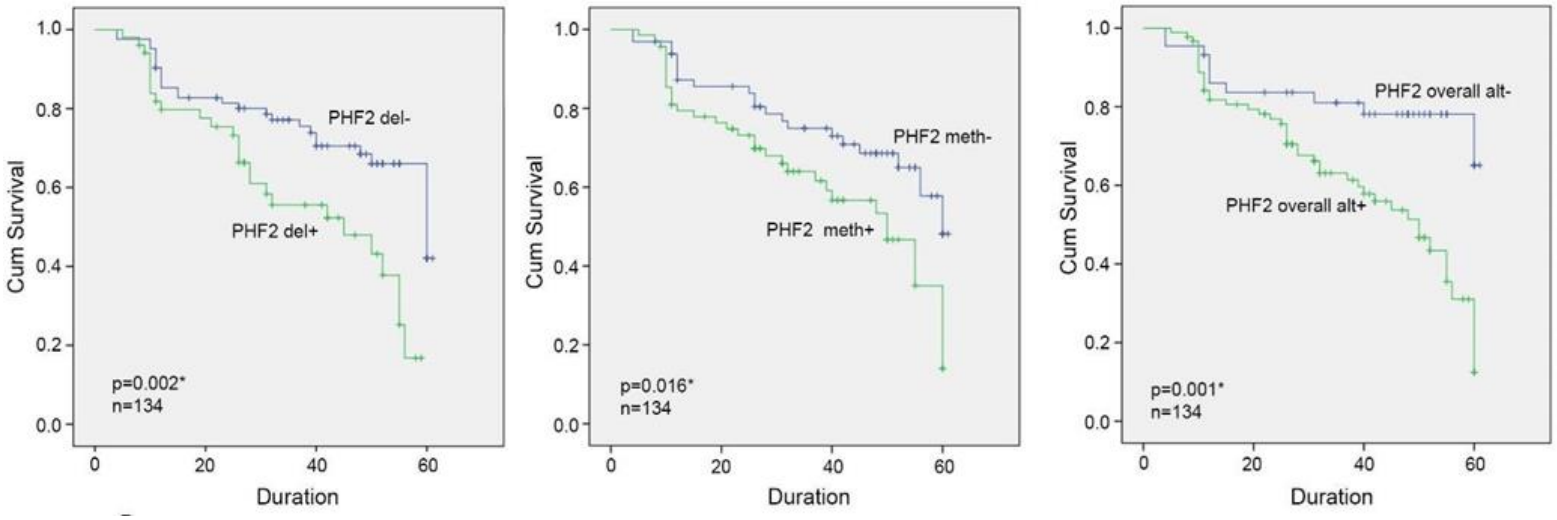


Figure 28: Survival analysis of the studied participants. Kaplan-Meier survival analysis curves in association with deletion, methylation and overall alteration of *PHF2*.

(3) **Inter-strand crosslink repair**

Genes involved: *FANCC*

FANCC in association with other FA (Fanconi anaemia) proteins forms a core complex to repair DNA damage (DNA inter-strand crosslink) at cell cycle checkpoints; failure of which leads to various consequences namely chromosomal instability, cell cycle alteration, and high sensitivity to oxidative damage and DNA crosslinking agents etc. Alterations of this gene has been reported in various clinical conditions like haematological neoplasia, solid tumor formation.[71,72] *FANCC* deletion has previously been reported in BLCA;[73] but its association with arsenic has not been studied yet as per our knowledge. In our study, we tried to figure out if there was any association of arsenic with genetic alterations of the locus.

Deletion analysis by microsatellite/ exonic markers:

High resolution CGH+SNP array data showed 40% (4/10) deletion in the bladder tumor samples included in the study. 60% (3/5) and 20% (1/5) of deletion were detected in AsH and AsL group, respectively (Figure 29A). Furthermore, validation of this array data by CNV analysis showed 33% (44/134) of deletion for *FANCC* locus. No significant association of *FANCC* deletion was obtained with arsenic content in the tissue (AsH vs AsL, 29%; 22/75 vs 37%; 22/59, p=0.358). Similar insignificant result persisted with tumor stages also (NMIBC vs MIBC, 32%; 28/87 vs 34%; 16/47, p=0.848) (Figure 29B).

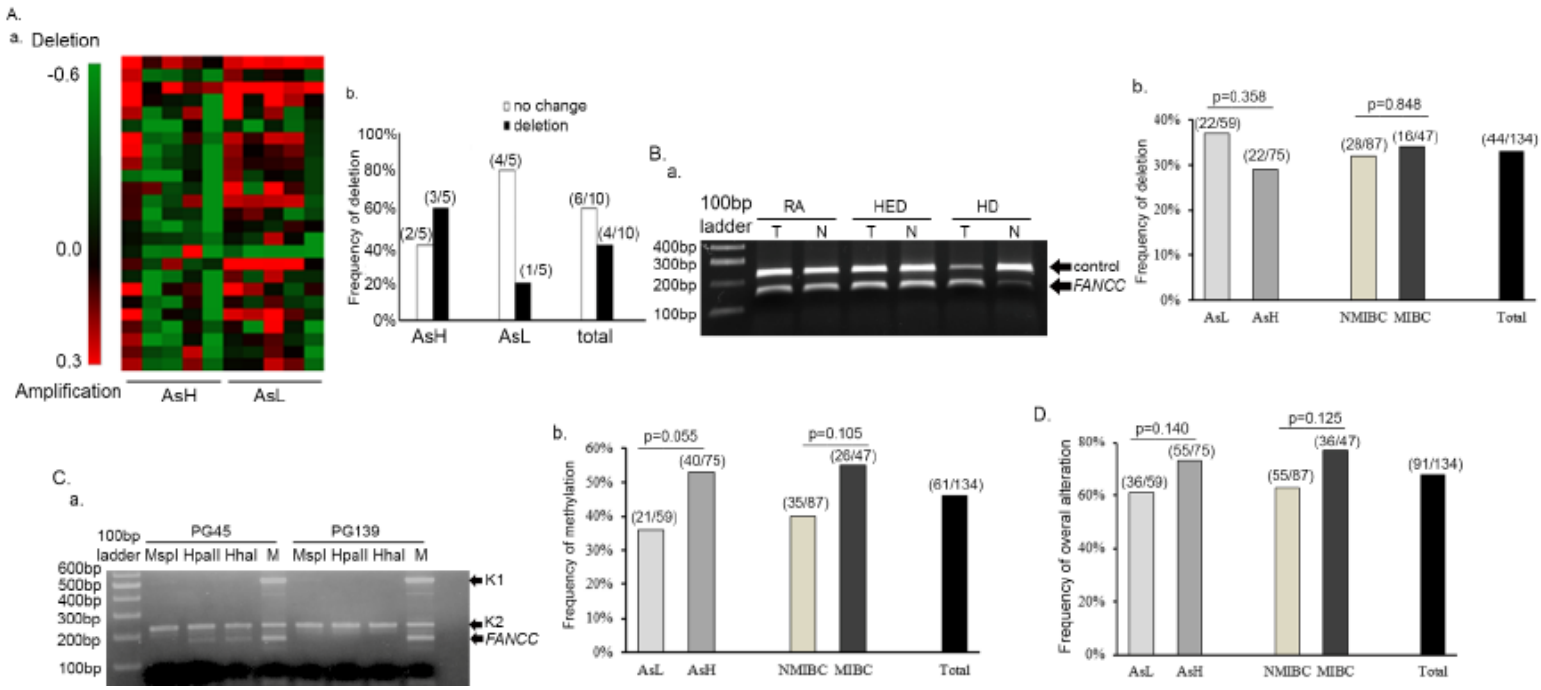


Figure 29: Molecular alteration status of *FANCC* locus. (A) Heat-map demonstrating deletion of AsH and AsL samples analysed in CGH+SNP array (a), histogram showing the summarized view of the array data (b). (B)

Representative gel image of CNV analysis of *FANCC* with types of alterations found (a), frequency of deletion in both AsH-AsL and NMIBC-MIBC groups (b). (C) Representative gel image of MSRA analysis of *FANCC* showing the status of methylated and unmethylated samples (a), frequency of methylation (b) and (D) Overall alteration in both AsH-AsL and NMIBC-MIBC groups.

Promoter methylation analysis by MSRA:

The gene was found to be methylated in 46% (61/134) of samples at its promoter CpGs. Promoter methylation frequency was marginally higher in AsH samples compared to AsL (53%, 40/75 vs 36%, 21/59; p=0.055) ones. Although, MIBC tumors showed much higher frequency of methylation than NMIBC, the difference did not attain statistical significance (55%, 26/47 vs 40%; 35/87, p=0.105) (Figure 29C).

Overall alteration frequency of the gene:

68% (91/134) of overall alteration (deletion/ methylation) of *FANCC* locus was seen in the bladder tumor samples. The frequency was increased with tumor invasiveness (NMIBC vs MIBC; 63%, 55/87 vs 77%, 36/47; p=0.125) and higher level of arsenic accumulated in the tissue (AsH vs AsL, 73%; 55/75 vs 61%; 36/59, p=0.140), but the differences were not statistically significant (Figure 29D).

mRNA expression analysis:

mRNA expression analysis of *FANCC* was done by qRT-PCR in 38 bladder tumor samples; of which 20 samples belong to AsH and 18 belong to AsL categories. Specificity of the reaction was checked in agarose gel to get the desired amplicon size. *FANCC* was shown to be reduced at mRNA level in 68% (33/38) of the bladder tumors. No significant association of the mRNA level expression of the studied gene was found with the tissue arsenic concentration and molecular alterations of the locus (p=0.889 and, 0.225, respectively) (Figure 30).

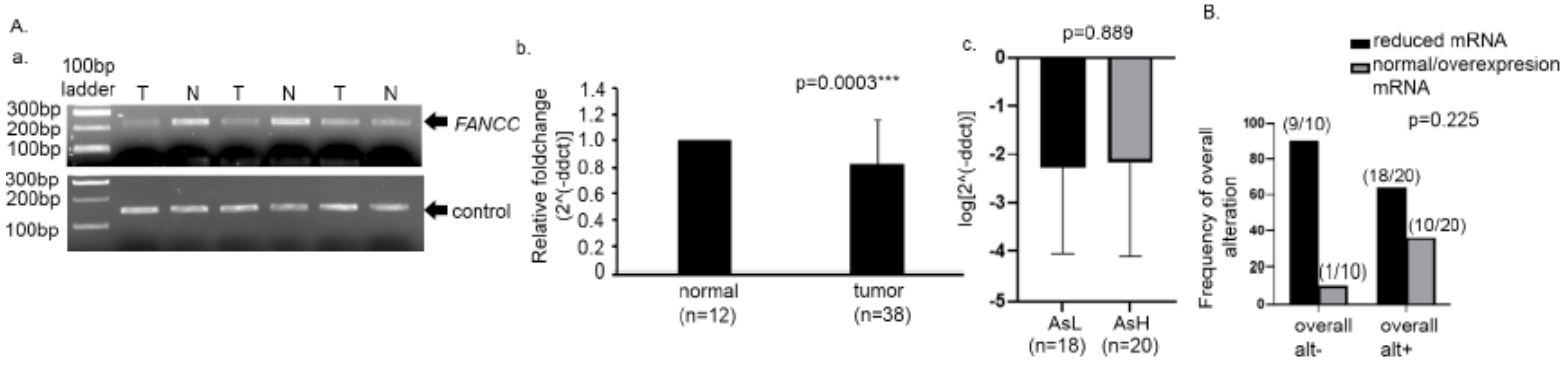


Figure 30: expression pattern of *FANCC* at mRNA level. (A) Representative gel image of mRNA expression of *FANCC* (225bp) with internal control, *B2M* (143bp) (a), histogram of the three genes showing fold change of each samples studied compared to adjacent normal urothelium (b), association of tissue arsenic with mRNA expression of the gene (c). (B) Association of mRNA expression of the gene with their overall molecular alteration.

(4) **Negative regulation of smoothened signaling pathway**

Genes involved: *PTCH1*

PTCH1 acts as a regulator of sonic hedgehog signalling by inhibiting SMO protein; in presence of Shh ligand, *PTCH1* inhibition is removed resulting into nuclear localization of GLI and subsequent transcription initiation of downstream genes involved in cell proliferation, apoptotic suppression etc. Absence of *PTCH1* makes the pathway to remain constitutively "on" resulting in uncontrolled proliferation of cells and other abnormality related to cancer. Dysregulation of *PTCH1* functioning has been studied in different cancers including bladder carcinoma.[74,75,76,77]

Our aim was to study the genetic and epigenetic alteration of *PTCH1* in BLCA patients that were being exposed to arsenic through drinking water leading to accumulation of arsenic in the bladder tissue.

Deletion analysis by microsatellite/ exonic markers:

Results of high-resolution array data suggested 30% (3/10) deletion of *PTCH1* gene. The deletion frequency were 40% (2/5) and 20% (1/5) for the AsH and AsL tumors included in the analysis (Figure 31A). Validation of array data was done by CNV analysis using both microsatellite and exonic markers in 134 tumor samples. The gene harbours a trinucleotide (CGG) repeat in the first intronic sequence that were found to be deleted often during tumorigenesis. Data of both microsatellite and exonic marker- based analysis were then combined to obtain the deletion status of the gene. 43% (58/134) of the tumors showed deletion for *PTCH1* locus. Deletion frequency was comparatively higher in AsL samples than AsH ones (53%; 31/59 vs 36%; 27/75, $p=0.078$); but the result was very much proportional between NMIBC and MIBC tumors (43% vs 43%, $p>0.9999$) (Figure 31B).

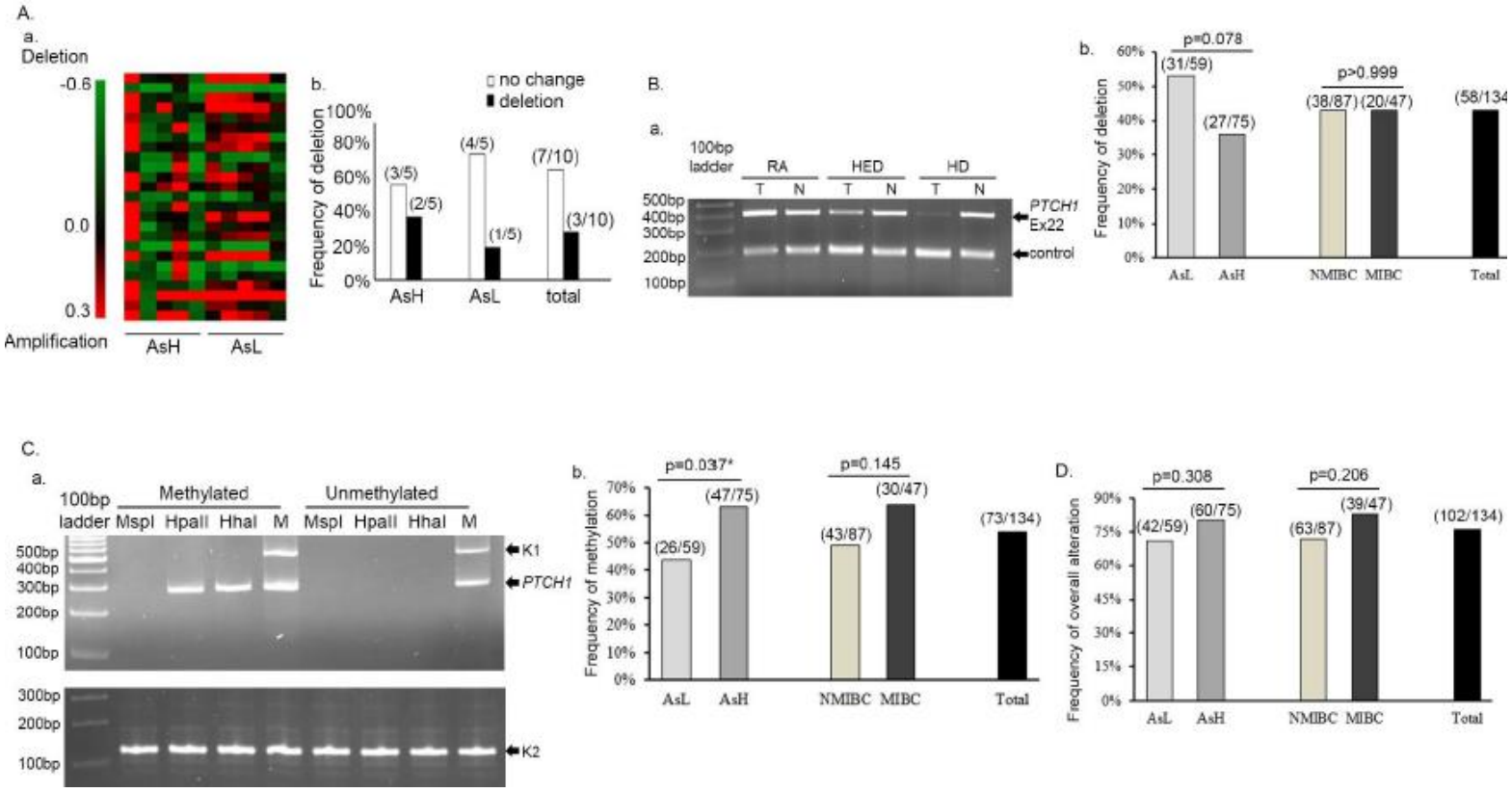


Figure 31: Molecular alteration status of *PTCH1* locus. (A) Heat-map demonstrating deletion of AsH and AsL samples analysed in CGH+SNP array (a), histogram showing the summarized view of the array data (b). (B) Representative gel image of CNV analysis of *PTCH1* with types of alterations found (a), frequency of deletion in both AsH-AsL and NMIBC-MIBC groups (b). (C) Representative gel image of MSRA analysis of *PTCH1* showing the status of methylated and unmethylated samples (a), frequency of methylation (b) and (D) Overall alteration in both AsH-AsL and NMIBC-MIBC groups.

Promoter methylation analysis by MSRA:

54% (73/134) of the tumors showed promoter methylation of *PTCH1* gene. Methylation frequency was significantly higher in AsH tumors compared to AsL (63%, 47/75 vs 44%, 26/59; p=0.037) but the difference is comparable between NMIBC and MIBC groups (49%, 43/87 vs 64%, 30/47; p=0.145) (Figure 31C).

Overall alteration frequency of the gene:

Although very high overall alteration of the gene (76%; 102/134) was observed in bladder tumor samples included in our study, the result failed to indicate any significant association with arsenic (AsH vs AsL, 80% vs 71%, p=0.308). The trend remained consistant with the muscle invasive properties of the tumor (NMIBC vs MIBC, 72% vs 83%, p=0.206) (Figure 31D).

mRNA expression analysis:

PTCH1 showed reduced expression at mRNA level in 84% (35/38) of the bladder tumors. Specificity of the reaction was checked by the presence of amplicon in agarose gel. No significant difference of mRNA expression was found between 20 AsH samples and 18 AsL used in the study (p=0.603) (Figure 32A). Interestingly, no significant association of the mRNA level expression of the studied genes was found with its molecular alterations also (Figure 32B).

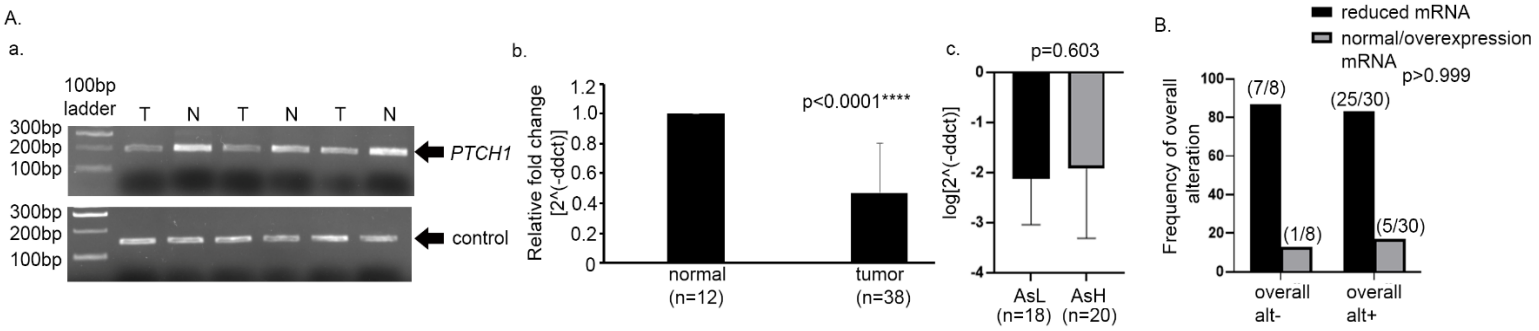


Figure 33: Expression pattern of *PTCH1* at mRNA level. (A) Representative gel image of mRNA expression of *PTCH1* (186bp) with internal control, *B2M* (143bp) (a), histogram of the gene showing fold change of each samples studied compared to adjacent normal urothelium (b), association of tissue arsenic with mRNA expression of the gene (c). (B) Association of mRNA expression of the genes with their overall molecular alteration.

Discussion:

People of West Bengal, living alongside the Gangetic belts were exposed to arsenic mainly through water used for drinking purpose.[78] Present study documented higher number of patients from arsenic-affected blocks/wards than arsenic safe ones, and higher tissue arsenic level was also measured in respective tumors. It suggested arsenic exposure as a major risk factor of BLCA in population of West Bengal; similar finding was reported in arsenic-endemic areas of Taiwan.[10] Furthermore, a strong correlation of tissue arsenic with arsenic concentration of drinking water samples and, underground water arsenic content record used for drinking was

found; this indicated contaminated drinking water as the major source of arsenic in studied population. In our study, bladder tumors with Ki67 overexpression (indicator of proliferation potential of tumor) showed significantly higher arsenic concentration than tumors with normal Ki67 expression; this provided the evidence of mitogenic insult of arsenic during bladder tumorigenesis. Additionally, the importance of arsenic in disease progression was evident from its gradual and significantly increasing concentration in tumors of higher pathological stages (Ta vs T1 vs T2 vs T3 + T4; NMIBC vs MIBC); however, the association of tobacco smoking, a known risk factor of bladder cancer, was not established in this connection. Also, in our sample pool, a higher proportion of muscle-invasive stages (T2/T3/T4) in AsH tumors than AsL type irrespective of tobacco habit (T+, As-L vs T-, As-H and T+, As-H) seemed to impose more importance on arsenic than tobacco in disease progression. Consistent to our data, a significant association of adverse tumor clinicopathological parameters was found with bladder cancer patients from arsenic exposed area than those from arsenic safe region.[79] In addition to progression, prognostic significance of arsenic was also evident in our study from its (arsenic) association with poor patient survival. Actually, in India, conventional therapies for bladder cancer include BCG and radical cystectomy followed by radiotherapy in NMIBC and MIBC respectively. On the background of immunosuppressive influence and ability to suppress radio-sensitivity, high arsenic level in tumor might interfere the host's immune response during BCG therapy and might induce resistance to cells against radiotherapy resulting treatment specific poor outcome of patients.[112]

To decipher the molecular pathogenesis of arsenic induced bladder cancer, we have focused on the deleted regions of chromosome-9 due to reported association of the chromosome with the disease condition in previous studies.[51,22,23] Chromosomal (Chr.9) regions exclusively deleted in AsH tumors were identified, the constituent genes (established/potential TSGs) were subjected to the pathway analysis and, ultimately some of the differentially altered molecular pathways were selected for validation based on their reported association with tumorigenesis. *CDKN2A* and *CDKA2B* loci encode p16, p14 and p15 proteins that regulate G1/S checkpoint negatively through CDK4/6 inhibition and stabilization of P53. The gene loci belong to a deletion prone region of the short arm of chromosome 9 (9q22).[64] High frequency of deletion of *p16*/*p14* (*CDKN2A*) and *p15* (*CDKN2B)* in primary bladder tumors of our study was in line with previous reports.[80,81] Interestingly, when arsenic was considered in the association study, a preferential deletion of *CDKN2A* was seen in AsH compared to AsL samples of array-based

data; however, in validation experiment by CNV analysis no additional impact of arsenic was seen on gene deletion in a comparative large and independent sample set. Like deletion, frequent methylation of *p16*, *p14* and *p15* genes promoter was reported in bladder cancer by independent investigators.[82,83,84] Similarly, high frequency of promoter methylation of *p16*, *p14* and *p15* was also seen in our study; but, very high and comparable methylation frequency between our AsH and AsL samples indicated no significant impact of arsenic on epigenetic alteration of these genes. Additionally, for the first time in our study, *p16*, *p14* and *p15* genes were analysed on the same set of bladder tumor samples including both NMIBC and MIBC; the overall alteration (deletion and/ or methylation) frequency of each gene was found to be about 80% in non-muscle invasive tumors, that (alteration frequency) remained fairly constant in muscle invasive samples also. Therefore, the inactivation of *CDKN2A* and *CDKN2B* loci seemed to be independent of arsenic exposure and an early event in the malignant transformation of normal urothelium. Similar to us, inactivation of *p15* was suggested to be an early event in bladder tumorigenesis by Frere-Belda et al (2001)[81]; but the inference was drawn from findings on superficial tumors (NMIBC) only and hence no comparison was done with data from muscle invasive tumors. Similar to molecular alterations, bladder tumors of our study showing reduced expression (~80%) for p14, p16, and p15 mRNAs remained fairly distributed between NMIBC and MIBC, and, AsH and AsL groups. Reduced expression (mRNA/protein) of *p16*/*p14*/*p15* was frequently reported in bladder cancer;[81,85,86] but, in majority of these studies, the underlying molecular mechanism remained largely unexplored. In our study, the majority of alteration positive tumors showed concordant reduced expression for studied genes, indicating the importance of deletion and promoter methylation in transcriptional gene silencing. However, a small proportion of our sample pool was alteration negative and, instead, a reduced gene expression was seen in the majority, indicating the involvement of additional mechanisms apart from deletion and methylation in the inactivation of studied loci.

In conclusion, molecular alterations at *CDKN2A* and *CDKN2B* loci seemed to be an early event in bladder carcinogenesis. Deletion and promoter methylation seemed to be major mechanism of gene inactivation. However, the studied loci were found to be dysregulated equally in AsH and AsL tumors, indicating their importance in disease pathogenesis irrespective of arsenic exposure.

To explore the role of negative regulation of ERBB signalling in arsenic induced bladder carcinogenesis, we have analysed molecular alterations of *SH3GL2* as the key negative regulator and its target, *EGFR* on same set of samples.

In both array-based experiment and CNV analysis, about 50-60% of bladder tumors showed deletion at *SH3GL2* locus and, the deletion frequency was substantially higher in AsH tumors compared to AsL ones. Compared to deletion, promoter methylation frequency of *SH3GL2* was considerably low (~30%) and did not differ much between AsH and AsL tumors. Therefore, arsenic exposure seemed to influence deletion at *SH3GL2* locus during bladder tumorigenesis but, such influence was not evident on epigenetic alteration of the gene. However, when deletion and promoter methylation were considered together, the overall alteration frequency remained significantly higher in AsH tumors compared to AsL, but comparable between NMIBC and MIBC groups; therefore, the inactivation of *SH3GL2* seemed to be involved in the early stage of bladder carcinogenesis and showed an incidental preference for arsenic exposure. This is the first-time data of its kind to our best. The molecular alteration of *SH3GL2* has not been analysed so far in bladder cancer; however, in head and neck[87], breast[88], and non-small cell lung cancer (NSCLC),[89] high frequency of deletion at the gene locus was in line with our data; but, reports on promoter methylation showed variation in different malignancies i.e., in NSCLC[89], no significant methylation was found in the promoter of *SH3GL2*; but, in breast cancer[88] and head and neck squamous cell carcinoma (HNSCC)[87], promoter methylation of the gene was found and the frequency (36-44%) was comparatively higher than that of our data. Moreover, in HNSCC[87], similar to our finding, the *SH3GL2* inactivation was found to be associated with the premalignant dysplastic lesions representing the early stage of development of the disease.

Like molecular alterations, reduced mRNA level expression of *SH3GL2* in bladder tumors of our sample set did not differ significantly between NMIBC and MIBC groups; a similar trend of *SH3GL2* expression was found between tumor vs. normal (reduced) and NMIBC vs. MIBC (comparable) samples of GEO data set. At protein level, a significant association of low/intermediate (reduced) level of *SH3GL2* with its molecular alteration and, concordance between RNA and protein level expression indicated the impact of deletion and methylation upon the gene expression. In line with our data, decreased SH3GL2 mRNA expression in both superficial and invasive bladder tumors of Oncomine database and low-level expression of the protein in commercially available bladder cancer tissue was reported. However, in this study, mRNA and protein expressions were not analysed on same sample set and, the underlying molecular mechanism for decreased expression was not explored.[67] Apart from bladder cancer, reduced expression of *SH3GL2* was also seen in breast cancer[88], NSCLC[89], and HNSCC[87] and in these studies, deletion and promoter methylation of the gene were highlighted as the major contributing mechanisms.

The overexpression of EGFR protein was frequently seen in bladder cancer;[90.91,92] similar phenomenon was also found in malignancies of head and neck, beast, lung and so on.[93,97,94] *EGFR* gene amplification and its association with the protein level over-expression were reported in oral, lung, cervical, esophageal, and breast cancer by some investigators; [93,94,95,96,97] On the contrary, no association between gene copy number and its expression (protein) level was found by others. [98,99] In our study, EGFR (protein) overexpression was recorded in 57% (44/77) of bladder tumors; but we did not see any concordance between mRNA and protein levels expression. Moreover, very low frequency of amplification was found at the *EGFR* locus (3%, 5/134), showing no statistical association with its expression level (protein). Interestingly, in our study, hemizygous deletion of *EGFR* gene was found in 19% (26/134) of bladder tumors of which 4 samples showed over-expression of the protein. Similarly, EGFR overexpression in small cell carcinoma of bladder having no amplification [100] and, in oral cancer even harbouring hemizygous deletion at the gene locus was reported. [101] Therefore, in bladder cancer and other malignancies, EGFR overexpression might be regulated by some mechanism apart from gene amplification and its influence at the transcriptional level.

SH3GL2 was found to regulate *EGFR* expression at the protein level through mediating its endocytosis from the cell surface and degradation. In bladder cancer, although SH3GL2 mediated internalization of EGFR was evident in cell line-based experiment,[67] no detailed analysis on this subject involving primary tumors along with patient related data has not been done so far. In our study, no significant association of SH3GL2 protein was found with EGFR mRNA, but both showed a highly significant negative correlation ($r=-0.7$, $p<0.0001$) at the protein level. Therefore, reduced expression of *SH3GL2* due to deletion and promoter methylation in bladder tumors of our study might cause dysregulated internalization of EGFR leading to its overexpression; this was also evident from significantly higher EGFR protein overexpression (but not mRNA) in AsH tumors compared to AsL against the background of significantly reduced SH3GL2 (mRNA and protein) expression in the former sample group than the latter. Moreover, the prognostic importance of arsenic induced inactivation of *SH3GL2* and associated EGFR overexpression was noticeable from our Kaplan-Meier and Cox regression analyses and, this seemed to provide a molecular basis for poor and hazardous outcomes of patients having high arsenic level in tumor shown previously. Similarly, EGFR (protein) overexpression due to deletion/ promoter methylation mediated inactivation of *SH3GL2* and, the prognostic significance of the phenomenon was reported in primary tumors of head and neck.[87]

In conclusion, arsenic induced inactivation of *SH3GL2* seemed to play an important role in the early stage of bladder carcinogenesis. *SH3GL2* inactivation might cause impairment in the EGFR homeostasis mechanism leading to its overexpression as seen in our tumor samples. Considering the prognostic significance of *SH3GL2* and *EGFR*, a detailed study of the pathway is warranted in bladder tumor to develop clinically relevant disease biomarkers and identify novel therapeutic targets.

PHF2 had been characterized as a novel histone H3K9 demethylase containing plant homeodomain- (PHD) and Jumonji C domain- (JmjC).[103] Role of *PHF2* in histone lysine demethylation and its associated transcriptional regulatory mechanism in bladder cancer is not explored till date. For the first time, our group has reported 38% deletion and 51% promoter methylation of *PHF2* in bladder tumors. Both deletion and promoter methylation frequencies were significantly higher in AsH tumors compared to AsL ones. Reduced expression of *PHF2* at both mRNA and protein level and its significant association with molecular alterations of the gene and with higher tumor arsenic level was found in our studied samples. It suggested a potential role of arsenic in alteration of *PHF2* locus in tumorigenic process. A previous study reported 42% deletion and comparatively lower promoter methylation (21%) of the gene with reduced expression of mRNA in breast cancer.[68] No report on molecular alteration of *PHF2* in bladder cancer was found till date. Deletion, promoter methylation, and expression of the gene (mRNA and protein) showed no significant difference between NMIBC and MIBC tumors which indicated altrations of *PHF2* to be an early event in bladder cancer development. Presence of promoter methylation in tumors along with its adjacent normal tissue were observed in our studied samples. Previous studies demonstrating promoter hypermethylation in tumor and adjacent normal tissues on breast[104] and colorectal cancer[105] suggested poor prognostic outcome in patients. Moreover, in our sample set, a distinct nuclear expression pattern of *PHF2* was evident in bladder tumor. A group has reported significant association of low level nuclear expression of *PHF2* with larger tumor size, advanced tumor stages and poor patient outcome and suggested *PHF2* as an independent prognostic factor in clear cell renal cell carcinoma (ccRCC).[106] Similar trend of result obtained from our study may be used to predict prognosis of the disease but detailed study in this regard is urgently required. In functional analyses, we found that *PHF2* down-expression was closely associated with reduced expression of *p21* compared to its own molecular alteration (*p21)* and expression of p53 protein, a transcription factor for *p21*. Similar to our findings, a study on colon and stomach

cancer reported a significant downregulation of *PHF2* that was positively associated with p21 down expression in presence of functional p53; it suggested the role of *PHF2* in *p53*-mediated transcriptional regulation of downstream target, *p21* that mediates cell apoptosis, by removing H3K9 methylation from *p21* promoter.[70]

However, for the first time our study has reported the detailed molecular alterations of *PHF2* in bladder cancer. It also established a strong association of tumor arsenic with the alterations of the gene. Therefore, the result of our analyses suggested that *PHF2* alteration in arsenic-contaminated area could be a major risk factor in development of BLCA and be used as potential marker of the disease for future studies.

FANCC acts as a core component of FA/BRCA pathway that involves in repairing interstrand DNA crosslink where lesions on two DNA strands joins covalently leading to impaired replication and transcription.[107] Failure of FA/BRCA pathway leads to developmental disorder as well as provide increased risk of cancer in organs like oral, oesophagus, brain etc.[108] *PTCH1* generally acts as a negative regulator of classical hedgehog (HH) signalling by suppressing *GLI* transcription factor which was found to positively regulate K-Ras, TGF-β, PI3K-AKT, and PKC-α pathways.[109,110] Chromosomal locus (9q22.3) containing FANCC and PTCH1 gene was found to be deleted in bladder cancer in several studies.[23,25] A detailed analysis of deletion/methylation analysis of the genes in bladder tumor is lacking. Our study reported 33% deletion and 46% methylation of *FANCC* locus in primary bladder tumor tissue; the frequency of deletion and methylation of *PTCH1* were 40% and 54%, respectively. But, the alteration did not show any association with tumor tissue arsenic. Although a large number of samples showing reduced expression at mRNA level showed molecular alterations at gene level, the association of the both did not attain statistical significance. Therefore, some additional factors, along with gene alterations, might play a role in regulating the expression of the genes. Argen et al.[111] reported that transcript of *PTCH1* was regulated by GLI transcription factor and other components of HH signalling (SHH and SMOH). An inactivating mutation of *FANCC* gene in HCC cell line has been reported by a group.[71] However, downregulation of both the genes are independent from the influence of arsenic exposure in our studied samples, at least. Further detailed investigation to make any conclusion in this regard is needed.

Significance of the study:

Aim of our study was to make a documentation of incidence of bladder cancer from different districts of West Bengal. Arsenic content of the tissue were measured in bladder tumor tissue and we were able to set a cut-off as permissible limit of arsenic in soft tissue (100ppb). The source of arsenic accumulated in the tissue is non-occupational and came predominantly through arsenic-contaminated drinking water. Arsenic can act as a mitogen in human tissue and associated with higher proliferation potential, adverse tumor stages. It can also serve as potential indicator for disease progression. We were also able to suggest some potential molecular markers that showed significant alteration in AsH tumors and could also be used as prognostic markers.

Future directions:

Diagnosis of bladder cancer is mainly dependent on cystoscopy, endoscopic resection of tumor followed by tumor staging, urine cytology etc. All these existing techniques are of invasive type and lacks accuracy. Lack of appropriate marker for population-based screening of the disease is another important issue. In our study, a group of molecular markers were identified to be altered irrespective of the presence of arsenic. The group of markers can be studied in independent paired (tumor tissue and exfoliated cells from urine) sample set. The set of markers found to be deregulated in both tumor and exfoliated cells can be used in case-control based study for further validation. Markers altered exclusively in case samples and not in the controls should be analysed for ROC curve in every possible combinations to get the maximum sensitivity and specificity. The best possible combination of markers resulted from ROC curve analysis can be used as diagnostic purpose for population-based non-invasive screening for early detection and better management of bladder cancer.

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Arsenic exposure and prognosis of bladder cancer in a patient population from West Bengal: an association study

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Arsenic level in bladder tumor of patients from an exposed population: association with progression and prognosis

Sabnam Ghosh[1], Mukta Basu[2], Kalyan Banerjee[1], Snigdha Pal Chaudhury[3], Tanmoy Paul[4], Dipak Kumar Bera[5], Dilip Kumar Pal[6], Ugir Hossain Sk[4], Chinmay Kumar Panda[1,7] & Amlan Ghosh*[1,8]

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Aims: To elucidate the impact of arsenic on progression and prognosis of bladder cancer. **Patients & methods:** Total arsenic in 145 tumors (80 non-muscle-invasive [NMIBC] and 65 muscle-invasive bladder cancer [MIBC]) was measured and associated with KI67 expression, tumor-clinicopathological parameters and patient outcome. **Results:** Tumor arsenic concentration was higher in exposed than unexposed patients (256 µg/kg vs 77 µg/kg; $p < 0.0001$) and positively correlated ($r = 0.65$; $p < 0.0001$) with arsenic content of patient's drinking water. Arsenic concentration showed significant association with KI67-overexpression ($p = 0.001$) and advanced tumor stages (NMIBC vs MIBC; $p = 0.0009$). In NMIBC, high tumor arsenic (>100 µg/kg) and KI67 overexpression was established as predictors for recurrence (hazard ratio [HR]: 4.68; $p = 0.005$ and HR: 3.91; $p = 0.018$) and progression (HR: 6.04; $p = 0.023$ and HR: 6.87; $p = 0.013$). In MIBC, association of high arsenic remained significant with increased risk of recurrence (HR: 4.58; $p = 0.04$). **Conclusion:** In NMIBC, high arsenic and KI67 overexpression and in MIBC, only high arsenic showed prognostic importance in predicting poor patient outcome.

Lay abstract: Research work suggests arsenic as risk factor for bladder cancer. In developing countries such as India, arsenic contamination of underground drinking water is a major health problem. The present study aimed to evaluate impact of arsenic on parameters of bladder cancer aggressiveness and clinical outcome of patients from West Bengal, India. Our data showed accumulation of arsenic in bladder tumor of patients exposed mainly through contaminated drinking water. Arsenic content in tumor favored aggressive phenotypes in bladder cancer (higher cell proliferation and advanced tumor stages) and was found to be a potential predictor for cases of death and disease recurrence in patients after receiving primary treatment measures. Therefore, arsenic content in bladder tumor may be used to improve existing protocols for better prediction of patient outcomes in populations with similar type of exposure.

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Keywords: bladder cancer • patient outcome • proliferation potential • tumor arsenic • West Bengal

Bladder cancer ranks among the top ten most common types of cancer globally [1], and in India, its overall incidence rate is 2.25% annually [2]. Bladder cancer is a disease of middle-aged to older adults with a long history of exposure to arsenic [3,4]. Arsenic is a major risk factor of bladder cancer; this was evident from arsenic-induced malignant transformation of human urothelial cell line [5] and overlapping incidence of the disease with arsenic-induced black-footed disease in endemic areas [6]. Nonoccupational exposure of arsenic is frequent and occurs mainly

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Integrative genomics and pathway analysis identified prevalent FA-BRCA pathway alterations in arsenic-associated urinary bladder carcinoma: Chronic arsenic accumulation in cancer tissues hampers the FA-BRCA pathway

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RAD50

ABSTRACT

Arsenic in drinking water is one of the major etiological factors in urinary bladder carcinoma (BCa). Here, high-resolution CGH-SNP microarray analysis in arsenic accumulated BCa tissues showed significant ($p < 0.05$) association of chromosomal alterations with high arsenic (≥ 112 ng/g) accumulation, further corroborated by high γH2AX nuclear expression. Cytobands 5q11–35, 9p24.3–21.3, 18q11.1–25, etc. showed deletion, whereas 12q was amplified in high arsenic samples (AsH). Consecutively, IPA® found FA-BRCA pathway to be exclusively altered in AsH group. Validation of several key regulatory genes (*RAD50*, *BRIP1*, *UIMC1*, *FANCD2*, *BRCA2* and *BRCA1*) of the pathway, were performed in independent BCa cases (n = 81). *UIMC1*, *RAD50* and *BRIP1* were differentially deleted and associated with poor survival of AsH samples. Moreover, reduced nuclear expression with diffused cytoplasmic expression of FANCD2 was higher in AsH samples. Collectively, frequent deregulation of *RAD50*, *UIMC1* and *BRIP1* may result in reduced nuclear translocation of FANCD2, which may cause more chromosomal aberrations among AsH samples.

1. Introduction

Urinary Bladder Carcinoma (BCa) ranks ninth worldwide [1] and the males are more affected than the females [4]. Broadly, BCa can be categorized into two major stages: a highly recurrent non-muscle invasive form (NMIBC) [Ta-T1] and a highly fatal muscle invasive form (MIBC) [T2-T4] [26]. Major risk factors like tobacco smoking, arsenic in drinking water and sometimes *Schistosoma* infection are reported to trigger the transformation of a normal urothelium [38,50]. Interestingly, in several developing countries, arsenic in drinking water was often shown to be associated with incidence and mortality of bladder cancer [23,27,29], [48].

Several studies over decades showed frequent loss of chromosomal regions like 1p22, 3p, 6q21, 7p21, 8p21, 9q, 11p13, 13q14, 17p13.2, 18q12 and frequent gain in chromosomal 1q22–25, 2p25, 4p16.3, 6p22, 8q21–22, 10p11–14, 12q13–15, 17q, 19q12, 20q11.2 and 22q11–13 regions in development of BCa [5,8,17,20, 41, 46]. Tobacco smoking has been reported to cause chromosomal instability in urinary bladder, colon, lung, breast etc. [7,44]. Amplification of 1p, 3q28, 5q11.2–12.1, etc. and deletion at 5q32, 13q, 17q21.31–21.33 etc. were reported to be markers for tobacco-induced chromosomal instability in Indian patients [9]. On the other hand, loss at 1q21.1, 7p22.3, 9q12 etc. were reported to be associated with arsenic exposure in lung squamous cell carcinoma among North-Americans [32]. However, chromosomal

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Original Manuscript

Divergent molecular profile of PIK3CA gene in arsenic-associated bladder carcinoma

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Abstract

The activation of *PIK3CA* in bladder carcinoma (BlCa) with its recurrent mutations in exon 9 and 20 were well reported. But the association of arsenic on the activation of the pathway is not well elucidated. Therefore, we aimed to analyse the effect of arsenic on the genetic (copy number variation/mutation) and expression profiles of *PIK3CA* in primary BlCa samples. Infrequent amplification (16%) of the *PIK3CA* locus was observed, with higher frequency among the arsenic-high (AsH) than arsenic-low (AsL) samples. Frequent (54%) tumour-specific mutations in exon 9 and 20 of *PIK3CA* were observed in the BlCa samples with prevalent (47%) C>T transition mutations. Exon 9 and 20 harboured 48% and 73% of the total mutations, respectively, with 37% in E542K/E545K and 25% of the mutation in H1047Y/R. Though mutation frequency in AsH and AsL was found to be comparable, we observed some arsenic-specific mutation at c.1633G>A, c.1634A>C (E545K) and c.2985C>T and c.3130G>T mutations, as well as prevalent transverse mutations of A>C and G>T in AsH group. Furthermore, 73% of the BlCa samples showed overexpression (mRNA/protein) of PIK3CA with genetic alterations (amplification/mutation), significantly ($P = 0.01$) higher in AsH group. However, 36% of the samples showed overexpressed PIK3CA, independent of mutation or amplification, signifying a transcriptional upregulation of *PIK3CA* gene. Therefore, the expression status of NFκB, a transcription factor of PIK3CA, was assessed and found to be significantly correlated with the overexpression of PIK3CA (mRNA/protein) in AsH group. Similarly, the expression pattern of pAKT1 (Thr 308) was also found to be significantly correlated with PIK3CA overexpression. Finally, AsH patients with the overexpression of PIK3CA or NFκB had the worst overall survival, signifying a strong impact of arsenic on this pathway and outcome of the patients. Thus, our study showed that the arsenic-associated differential molecular profile of PIK3CA/AKT1/NFκB in BlCa has an important role in the molecular pathogenesis of the disease.

Introduction

Urinary bladder carcinoma (BlCa) ranks ninth worldwide and sixth among the males (1). The disease progresses from early stages, i.e. non-muscle-invasive type (NMIBC), Ta–T1, to later stages, i.e. muscle-invasive type (MIBC), T2–T4 (1). Tobacco smoking, chemical exposure to carcinogen, hormonal effect, arsenic in drinking water and *Schistosoma* infection remain some of the major aetiological factors (2,3). It was evident that arsenic in drinking water above World

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ORIGINAL ARTICLE – CANCER RESEARCH

High nuclear expression of HIF1α, synergizing with inactivation of LIMD1 and VHL, portray worst prognosis among the bladder cancer patients: association with arsenic prevalence

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Abstract

Purpose Our study was aimed to understand the importance of LIMD1-VHL-HIF1α pathway in development of bladder carcinoma (BlCa) in association with arsenic prevalence.

Methods At first, the mRNA expression pattern of the genes of this pathway (LIMD1, VHL and HIF1α) was checked in GEO datasets and in our samples. Next, genetic and epigenetic profiling of LIMD1 and VHL was done in our sample pool, validated in T24 BlCa cell line. The results were next correlated with various clinico-pathological parameters.

Results Differential under-expression of LIMD1 and VHL genes was found in muscle-invasive BlCa (MIBC) in comparison to non-muscle-invasive BlCa (NMIBC). However, HIF1α protein, but mRNA, was found to be overexpressed among the MIBC samples; depicting the probability of HIF1α protein stabilization. Analysis of genetic and epigenetic profiles of LIMD1 and VHL exposed a frequent promoter methylation of LIMD1 gene in MIBC samples. Further, in-depth look into the results unveiled that the high nuclear expression of HIF1α was significantly correlated with genetic alterations of LIMD1, alone or in combination with VHL. Moreover, treating the T24 cells with a de-methylating agent (5-aza-2′-deoxycytidine) re-expressed the methylated LIMD1 and VHL genes, which in turn, reduced the HIF1α protein level significantly. Additionally, patients with high arsenic content (> 112 ng/g, AsH) seemed to have recurrent promoter methylation in LIMD1, as well as co-methylation/alteration of LIMD1 and VHL gene. Lastly, high nuclear expression of HIF1α in association with co-alteration of VHL and LIMD1 showed the worst overall survival (OS) among the patients.

Conclusion To conclude, MIBC samples portrayed higher alterations in VHL and LIMD1, thereby, stabilizing HIF1α protein and lowering the OS of patients.

Keywords LIMD1 · VHL · HIF1α · Kaplan–Meier survival analysis · Bladder carcinoma

Introduction

Urinary bladder carcinoma (BlCa) is the ninth-most common occurring cancer worldwide (Bray et al. 2018). Early stages or non-muscle-invasive carcinoma (NMIBC; Ta-T1) has better prognosis than late stages or muscle-invasive carcinoma (MIBC; T2-T3) (Bray et al. 2018). Therefore, molecular pathways related to metastasis or angiogenesis or hypoxia could serve as essential driver pathways for BlCa progression. Till date, several molecular pathways have been postulated to accelerate the disease to later stages and hence resulted into poor prognosis (Mitra et al. 2006). Common pathways among them are Ras-MAPK, p53 cell cycle regulation pathway, retinoblastoma pathway, etc. (Mitra et al. 2006; Williams and

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Differential operation of MLH1/MSH2 and FANCD2 crosstalk in chemotolerant bladder carcinoma: a clinical and therapeutic intervening study

Mukta Basu[1,4] · Debalina Mukhopadhyay[1] · Balarko Chakraborty[1] · Sabnam Ghosh[2] · Dilip Kumar Pal[3] · Amlan Ghosh[2] · Chinmay Kumar Panda[1]

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Abstract
We aimed to understand the crosstalk between mismatch repair (MMR) and FA-BRCA pathway in primary bladder carcinoma (BICa) samples as well as in chemotolerant cell line. We analysed the genetic alterations of *MLH1* and *MSH2* (MMR-related genes) and after that we correlated it with the nuclear translocation of FANCD2 protein. Next, we evaluated this crosstalk in T24 BICa cell line in response to doxorubicin treatment. In primary BICa tumors, infrequent genetic deletion (17–20%) but frequent promoter methylation (28–55%) of *MLH1* and *MSH2* was observed, where *MLH1* was significantly ($p < 0.05$) more methylated among the early staged samples (NMIBC). However, *MSH2* was significantly more altered among the NMIBC samples, signifying the importance of MMR pathway during the early pathogenesis of the disease. Furthermore, BICa samples with underexpressed MLH1/MSH2 protein possessed cytoplasmic FANCD2 protein; encouraging that inefficiency of MMR proteins might restrict FANCD2 nuclear translocation. Next, we analysed publicly available data in GEO2R tool where we observed that in response to chemotherapeutic drugs, expression of MLH1, MSH2 and FANCD2 were diminishing. Validating this result in doxorubicin tolerant T24 cells, we found that expression of MLH1 and MSH2 was gradually decreased with increasing dose of doxorubicin. Interestingly, FANCD2 mono-ubiquitination (L-form) was also reduced in chemotolerant T24 cells. The crosstalk between MMR and FA-BRCA pathway was substantiated in the primary BICa tumors. Further, in response to doxorubicin, this crosstalk was found to be hampered due to under-expression of MLH1 and MSH2 gene, thereby rendering chemotolerance.

Keywords Mismatch repair pathway · FA-BRCA pathway · MLH1 · FANCD2 · Chemotolerance

Introduction

Urinary bladder carcinoma (BICa) ranks ninth among all the cancer types and its mortality rate have been quite high in recurrent cases [1]. Several authors have shown huge burden of genomic and chromosomal instability among the BICa patients [2, 3], including TCGA Bladder cohort study where frequent missense mutations were found among several DNA repair pathway genes [4]. Further, insufficient expression of mismatch repair (MMR) pathway proteins like PMS2, MLH1 and MSH2 was positively correlated with poor disease-free survival and disease progression [5, 6]. However, insights into the genetic alterations in *MLH1* and *MSH2* are still warranted in BICa.

There are a few studies which have shown altered expression of FA-BRCA pathway genes in BICa [7]. In FA-BRCA pathway, the key event for the pathway to function effectively

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Association of arsenic with recurrence of urinary bladder cancer

Dilip K Pal[1], Akash Agrawal[2], Sabnam Ghosh[3] and Amlan Ghosh[4]

Abstract

Arsenic is known to be an important aetiological factor for the development of urinary bladder cancer. It is known to be found excessively in ground water in certain geographical areas, including West Bengal. We have studied patients with recurrent bladder cancer from different areas of this Indian state and correlated arsenic as a causative aetiological factor for development and aggressiveness of the biological behaviour of urinary cancer. We included 31 patients from various parts of West Bengal state with recurrent bladder cancer who were operated in our institute. Their clinical and residential data and their arsenic content of tumour tissue were measured. Statistical analysis was performed to test the association of tissue arsenic with clinicopathological features of recurrent disease. We found very high levels of arsenic in tumour tissue in all residents of the districts with high prevalence of arsenic in the drinking water. We also observed more aggressive clinicopathological progression and early recurrence in patients with high arsenic content. We conclude that arsenic is a causal factor in the clinicopathological progression of recurrent urinary bladder cancer. Measures to decrease the level of arsenic in drinking water should be taken as this may both improve clinicopathological outcomes in the recurrence of urinary bladder carcinoma, as well as reducing its overall incidence.

Keywords

Arsenic, bladder cancer, recurrent bladder tumour, smoking

Introduction

Urinary bladder cancer is strongly associated with environmental exposure of carcinogens. This association is especially strong for arsenic, which has been shown to induce malignant transformation of the urothelium. It correlates to the endemic areas where populations were identified with arsenic-induced skin cancer.[1,2] Excess inorganic arsenic in drinking water from artesian wells is implicated as the commonest route of exposure,[3–5] though environmental, agricultural and occupational roles may also be significant. Distribution of arsenic in West Bengal is shown in Figure 1A.

According to a report submitted by the School of Environmental Studies, Jadavpur University, on the contamination of ground water in West Bengal, five districts (Bankura, Birbhum, Purulia, Medinipur East and Medinipur West) were unaffected or arsenic safe (arsenic concentration < 10 µg/L), five (Koch Bihar, Jalpaiguri, Darjiling, North Dinajpur and South Dinajpur) were mildly affected (arsenic concentrations 11–50 µg/L) and the remaining nine (Malda, Murshidabad, Nadia, North-24 Parganas, South-24 Parganas, Barddhaman, Haora, Hugli and Kolkata) were severely affected (arsenic concentrations > 50 µg/L).[6] This is shown in Figure 1.

Our study detects the presence of arsenic in tumour tissue in cases of recurrent bladder cancer and its relation to arsenic levels in the water at the home of the patient. We looked at the recurrence interval, stage progression, and grade of tumour and the association with the arsenic content in tumour tissue. Although tumour

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244, A.J.C. Bose Road, Kolkata – 700020.
IPGME&R Research Oversight Committee
(Institutional Ethics Committee)

Memo No. IPGME&R/IEC/2019/107 Date: 06.02.2019

Dr. Amlan Ghosh
Assisstant Professor
Department of Biological Science
Presidency University, Kolkata

Dear Dr. Ghosh,

A meeting of the Institutional Ethics Committee of IPGME&R, Kolkata, was held on 02.02.2019 at 12:00 Noon in the IPGME&R Dean's Office. In this meeting, the members considered the protocol of your project titled:

Identification of susceptible genes associated with arsenic induced bladder cancer in West Bengal, India.

The following additional documents were reviewed:

- Informed consent documents in English.
- Informed consent documents in Hindi.
- Informed consent documents in Bengali.

After deliberations and review the committee took the following decision regarding the project:

Approved

The Committee understands that this is an academic research project without any commercial sponsor and the project is proposed to be conducted as PhD research project of Presidency University, Kolkata, with Dr. Dilip Kumar, Professor & Head, Department of Urology, IPGME&R, Kolkata, as the local supervisor.

The Committee further understands that this project has already received approval from us under the same title, vide our letter Inst/IEC/307, dated 27.05.2013, with Dr. Chinmay Kumar Panda, Department of Oncogene Regulation, Chittaranjan National Cancer Institute (CNCI), Kolkata, as the Principal Investigator (PI), in which you were the Co-PI. There has been no change in study objectives or methodology. However, the project work is still to be completed and a new student will continue the work as a PhD program under your supervision. The work is expected to continue at least till end of November, 2019.

It is placed on record that the decision regarding your proposal was unanimous and therefore did not require any voting procedure. The list of committee members present in this meeting are appended to this page. No members were absent in this meeting.

Additional points, if any, stated on the next page are to be noted.

Continued on Page 2

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IPGME&R Research Oversight Committee
(Institutional Ethics Committee)

Continued from Page 1

Additional points to be noted

- The Committee expects that any amendments to the Study Protocol, Informed Consent documents or other relevant documents would be brought to its notice.
- A brief project completion report is to be submitted to the IPGME&R Research Oversight Committee. If project duration exceeds 1 year from commencement, a brief annual progress report should also be submitted.
- IPGME&R Research Oversight Committee is registered with Central Drugs Standard Control Organization (CDSCO), Government of India, in consonance with Rule 122D of the revised Drugs & Cosmetics Rules 1945 – Registration No. ECR/35/Inst/WB/2013/RR-16. It functions in accordance with revised Schedule Y and Indian Council of Medical research (ICMR) guidelines.

List of institutional ethics committee members who attended the meeting on 02.02.2019

SN	Name & role in the committee	Gender	Designation
1	**Dr. Hemanta Kumar Majumder** [Scientist & Chairperson]	Male	Senior Scientist, Indian Institute of Chemical Biology, Kolkata
2	**Prof. Amal Kanti Das** [Pharmacologist]	Male	Dean of Student Affairs and Head, Department of Pharmacology, IPGME&R
3	**Prof. Biswanath Kahali** [Forensic Expert]	Male	Head, Department of Forensic Medicine, IPGME&R
4	**Prof. Swati Chakravarti** [Clinician – Pediatrician]	Female	Consultant, Institute of Child Health, Kolkata
5	**Prof. Jayanta Chatterjee** [Clinician]	Male	Former Head, Department of Nuclear Medicine, IPGME&R
6	**Prof. Bijay Kumar Majumdar** [Clinician]	Male	Former Head, Department of Plastic Surgery, IPGME&R
7	**Prof. Amal Kumar Santra** [Basic medical scientist]	Male	Scientist, Department of Gastroenterology, IPGME&R
8	**Prof. Biman Kanti Ray** [Clinician]	Male	Professor, Department of Neurology, Bangur Institute of Neuroscience (BIN), IPGME&R, Kolkata
9	**Dr. Bobby Pal** [Public health expert]	Female	Associate Professor & Head, Department of Preventive & Social Medicine, All India Institute of Hygiene & Public Health, Kolkata
10	**Mr. Debdut Mukherjee** [Legal expert]	Male	Advocate, Calcutta High Court
11	**Mr. Arunangshu Shekhar Jana** [Social worker]	Male	Social worker, Mahendraganj, Dist. South 24 Parganas
12	**Dr. Nila Majumdar** [Lay person]	Female	Bengali teacher, Kolkata
13	**Prof. Avijit Hazra** [Pharmacologist & Member secretary]	Male	Professor, Department of Pharmacology, IPGME&R

Avijit Hazra 06/02/2019

Dr. Avijit Hazra – Member Secretary
IPGME&R Research Oversight Committee

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